Cardiovascular Tonic Effects of Compound Formula of *Radix Salviae miltiorrhizae* and *Radix Puerariae*

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Abstract

Radix Salviae miltiorrhizae (known as Danshen) and *Radix Puerariae* (known as Gegen) have individually been used in Chinese medicine to treat coronary heart diseases particularly in myocardial infarction and hypertension. Therefore, the cardiovascular tonic effect of the compound formula of Danshen and Gegen is of interest to be examined.

In "Zhong yao fang ji xian dai yan jiu da dian" (1996), Danshen, Gegen and *Rhizoma corydalis* (known as Yanhu) are formulated in the weight ratio 6:3:1 in an old formula. Basing on this information, preliminary investigations were carried out. The cardiovascular tonic effect of the compound formula of Danshen (D) to Gegen (G) at 7:3 ratio was further studied. For the long-term cardiovascular protection, the effects of the compound formula on antioxidant, vasodilation, and anti-platelet formation were focused.

The antioxidant effect was investigated by 2 models, namely 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) induced red blood cell hemolysis and myocardial ischemia-reperfusion of isolated SD rat heart. IC50 of aqueous extract of 7:3 (D:G) compound formula in AAPH induced RBC hemolysis was 141 μg/ml. The 7:3 (D:G) compound formula protected ischemia reperfusion heart by exhibiting 60% contractile force recovery and reducing the heart damage which was reflected by heart specific enzyme LDH and CK assays.

The vasodilation effect was investigated by the model of isolated SD rat aorta rings. 7:3 (D:G) compound formula showed vasodilation in a dose dependent manner. The synergistic vasodilation effect of 7:3 (D:G) compound formula was shown when comparing with individual Danshen and Gegen. In aorta with removed endothelium and intact aorta treated with nitric oxide synthase inhibitor, N^G-nitro-L-arginine (L-NNA), the vasodilation effect of Gegen extract was found to be alleviated. This indicates that the vasodilation effect of Gegen extract is endothelium dependent and the mechanism is via the function of nitric oxide synthase.

The effect on the platelet formation was investigated by the colony forming unit - megakaryocyte plasma clot colony assay. The 7:3 (D:G) compound formula inhibited MK cell proliferation at IC50 of 161 μg/ml.

Capsules containing 500 mg aqueous extract of 7:3 (D:G) compound formula were manufactured in compliance to Good Manufacturing Practice (GMP) standard and double blind clinical trial with placebo was started in Prince of Wales Hospital, Hong Kong.

摘要

丹參及葛根單獨使用於治療冠心病尤其心肌梗塞及高血壓，所以丹參葛根複方的心血管保健作用引起了測試的興趣。

根據在 1996 年出版的《中藥方劑現代研究大典》中以重量比例 6:3:1 配制的複方丹參葛根元胡作爲參考，作了初步的測試後，以比例 7:3 配制的複方丹參葛根的心血管保健作用作進一步的研究。長期的心血管保健作用研究集中於複方的抗氧化作用、血管舒張作用及抗血小板形成作用。

複方丹參葛根(7:3)的抗氧化作用利用 2-2'-偶氮-二-(2-脒基丙烷)二氫氯化物(AAPH)引起的紅血球溶血作用及大鼠離體心臟缺血再灌注模式作測試。複方丹參葛根(7:3)對於 AAPH 引起的紅血球溶血作用具有抑制作用，半數抑制濃度(IC50)爲 141 μg/ml。複方丹參葛根(7:3)對大鼠離體心臟缺血再灌注損傷呈現保護作用，心肌收縮力恢復率達 60%並同時減低了心肌特異性酶—乳酸脫氫酶(LDH)及肌酸激酵素(CK)釋放於培養液內的的活性，証明保護心肌細胞破壞。

複方丹參葛根(7:3)的血管舒張作用是利用大鼠離體大動脈環模式作測試。複方丹參葛根(7:3)對血管舒張作用呈現劑量依賴性。與單方丹參及葛根比較，複方丹參葛根(7:3)呈現加成的血管舒張作用。葛根對於血管舒張作用在血管內皮去除組及一氧化氮合成酶抑制劑—N-硝基-L-精氨酸(L-NNA)處理組內被取消，所以葛根對於血管舒張作用的基理是依賴血管內皮及牽涉一氧化氮合成酶的功能。

複方丹參葛根(7:3)的抗血小板形成作用利用集落形成單位巨核細胞(CFU-MK)血漿凝塊集落培養模式作測試。複方丹參葛根(7:3)對於巨核細胞的細胞增生具有抑制作用，半數抑制濃度(IC50)爲 161 μg/ml。

依據優良產品製造規範(GMP)標準下生產了複方丹參葛根(7:3)的水提取物膠囊。每粒膠囊含有 500 mg 複方丹參葛根(7:3)水提取物。複方丹參葛根(7:3)膠囊的雙盲及具安慰劑的臨床研究正在香港威爾斯親王醫院進行中。

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AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
AChE	Acetylcholine esterase
CFU-MK	Colony-forming-unit megakaryocyte
CK	Creatine kinase
EDRF	Endothelial-derived relaxing factor
ET-1	Endothelin-1
g	Gravity
GAP	Good Agriculture Practice
GMP	Good Manufacturing Practice
HKIB	Hong Kong Institution of Biotechnology
HPLC	High-Performance Liquid Chromatography
IC50	50% Inhibition Concentrations
IMDM	Iscove's modified Dulbecco's medium
LDH	Lactate dehydrogenase
LDL	Low Density Lipoprotein
L-NNA	N^G-nitro-L-arginine
MK	Megakaryocyte
NO	Nitric oxide
NOS	Nitric oxide synthase
ODS	Octadecyl silica
PBS	Phosphate Buffer Saline
RBC	Red Blood Cell
SD rat	Sprague-Dawley rat
TCM	Traditional Chinese Medicine
TFA	Trifluoroacetic acid
TPO	Thrombopoietin
U46619	9,11-dideoxy-9α,11α-epoxymethano prostaglandin $F_{2\alpha}$
VSMC	Vascular smooth muscle cells

Chapter 1 Introduction

Traditional Chinese Medicine (TCM) is a powerful method of healing. They are natural. Formulae are available in a variety of forms such as crude herbs to be boiled into tea, liquid bottled extracts, ground herbs packaged in pills, and powders. Herbs more like foods than drugs, can supplement our diet and fortify our constitution.

They have fewer side effects when compared to Western medicine. Western drugs often control symptoms, but do not alter the disease process. TCM treats the underlying condition as defined by traditional diagnosis, and rarely causes unwanted side effects. Sometimes long-term use of herbs is desirable whereas extended use of pharmaceuticals would not be healthy.

TCM can cure but also prevent diseases. Herbal practitioners view people as ecosystems in miniature and seeks to improve one's capacity to balance and renew his resources. Often Western medicine intervenes only after crisis arises, whereas TCM anticipates problems by sustaining our interior landscape. By correcting depletion and stagnation at earlier stages, greater problems later on are avoided.

Sometimes Western medicine has nothing to offer for nagging chronic complaints that Chinese medicine can help. Western and Chinese medicines are not a substitute for each other and they are often complementary to each other. Whereas Western medicine may heroically rescue us, Chinese medicine can protect and preserve our health day to day.

This project is going to find out a cardiovascular tonic supplement from TCM. That supplement can gain all the advantages of TCM and at the same time overcome all the disadvantages such as, low potency, inconvenience and time consuming for taking TCM. These are some of the reasons that TCM products are not well accepted in Western countries.

Radix Salviae miltiorrhizae (Danshen, in Pinyin name) and *Radix Puerariae* (Gegen, in Pinyin name) are individually studied by many research groups. Some of their pharmacological effects are related to cardiovascular disease treatment.

Many research projects were done on Danshen, and a number of active constituents were identified, such as tanshinones I, tanshinones IIA, tanshinones IIB, protocatechualdhyde, salvianolic acid B and lithospermic acid. Danshen is known to have many pharmacological effects. It can lower blood pressure (Kang *et al.*, 2002), protect ischemic reperfused myocardium (Wang *et al.*, 1997), improve micro-circulation (Xue, 1986), act as anticoagulant (Onitsuka *et al.*, 1983), act as antioxidant (Wu *et al.*, 1998) and exert anti-tumor effect (Ryu *et al.*, 1997).

Gegen also has a number of identified active constituents, most of them are isoflavones or their derivatives, such as puerarin, daidzin, daidzein and genistein. Gegen is also known to have many pharmacological effects. It can act as a beta-adrenergic blocker (Feng et al., 1984), cause coronary (Fan *et al.*, 1982) and cerebral vasodilatation (Tseng *et al.*, 1974), lower blood pressure (Song *et al.*, 1988), inhibit platelet aggregation (Yang *et al.*, 1990) and exert anti-arrhythmic effect (Chai *et al.*, 1985), anti-pyretic effect (Fan *et al.*, 1997) and anti-alcoholism effect (Keung

and Vallee, 1998).

Chinese herbs are usually combined in formulae to enhance their properties and actions. Symptoms and signs are matched with therapeutic effects, reflecting the particular conditions and needs of each patient. Therefore, to examine a compound formula of Danshen and Gegen was of interest and its cardiovascular tonic effect was studied.

My studies focused on 1) working out a correct combination ratio for a better compound formula and 2) the effects of the formula on the cardiovascular system with emphasis on three major areas namely, antioxidant protection, vasodilation and anti-platelet formation. All this information is valuable for further study on this compound formula. The capsules of the compound formula were manufactured in compliance with Good Manufacturing Practice (GMP). The capsules were used in clinical trial study at Prince of Wales Hospital, Hong Kong.

Chapter 2 Establishment of Compound Formulation

We are interested to produce a compound formula consisting of Danshen and Gegen for cardiovascular tonic use. Therefore the first step of the project was to find out a correct ratio to combine these two TCMs. The strategy of finding out the correct combination ratio of Danshen and Gegen, way that, first finding out all the existing compound formulae which consist of both these TCMs, and then carrying out preliminary experiments to identify one single chosen compound formula.

2.1 Formulation research

In order to find out all existing compound formulae, information in "Zhong yao fang ji xian dai yan jiu da dian"(Huang and Shi, 1996) was scanned. There are 34 compound formulae being found to consist of both Danshen and Gegen. Only six of them are not cardiovascular tonic related. Among the 28 formulae, the most complicated one consists of Danshen, Gegen and 12 other TCMs, such as Ginseng, Sanqi, Chuanxiong. While the simplest one consists of only three TCMs, Danshen, Gegen and Yanhu.

Danshen-Gegen-Yanhu tablet is the simplest formula that consists of ground Danshen, Gegen and Yanhu in a ratio of 6:3:1 by mass in each tablet (0.6 g). It was used to treat coronary heart diseases in human and the dosage was four tablets a time, three times a day for four weeks (孫永智, 1986). Yanhu is also called Yanhusuo and its Latin name is *Rhizoma Corydalis*. Recent research shows that Yanhu has the pharmacological effects such as, analgesic effect (Hu *et al.*, 1994 ; Yue, 1981),

hypnotic effect (Buda *et al.*, 1984), sedative effect (Jin *et al.*, 1984), ulceration protective effect (Soji *et al.*, 1969) and muscle relaxation effect (Zhong *et al.*, 1986). A series of active constituents such as *d*-corydaline, *dl*-terahydropalmatine, protpine, corydalis *L* were identified too.

With reference to the information from this tablet, the compound formula of Danshen to Gegen to Yanhu was in a ratio of 6:3:1. As a recent research paper shows that Danshen plays a very good cardio-protective role (Ji et al., 2003), one part of Danshen was used to replace Yanhu to form a new ratio of Danshen to Gegen in 7:3 which was examined in our project. Ratio of Danshen to Gegen in 2:1 and 1:1 are more frequently found among the above mentioned 28 old compound formulae. Therefore, Danshen to Gegen in a ratio 2:1 and 1:1 were also tested for their possible cardiovascular tonic effect.

Therefore four ratios by weights are used in my study, namely, Danshen to Gegen to Yanhu in 6:3:1, Danshen to Gegen in 7:3, 2:1 and 1:1.

2.2 Aqueous extract preparation

The aqueous extracts of the chosen TCMs compound formulae were interesting because they would share more proprieties with the decoctions when compared to the old compound formulae. The powder form of aqueous extracts of the four focused compound formulae in appropriate ratios of Danshen, Gegen and Yanhu by weights were first prepared. And the aqueous extracts of the three individual TCMs were also prepared.

2.2.1 Materials and methods

Raw materials

Danshen was supplied by New World China Enterprises Projects Limited and the Danshen was grown at Sichuan province in compliance with Good Agriculture Practice (GAP). Gegen and Yanhu were purchased from a local herbal company in Hong Kong. They were cut into small pieces before extraction, where Danshen was cut into pieces of size 1 x 1 x 1 cm, Gegen was cut into pieces about 3 x 3 x 0.5 cm pieces and Yanhu was cut into 1 x 1 x 0.5 cm pieces.

Reflux extraction

Appropriate amount of the raw materials were weighed and then slightly washed with distilled water. Raw materials were refluxed with distilled water in 1:10 (weight (g) to volume (ml)) for two hours on a heating mantle. The time was started to count after water got boiled. After two hours reflux, the decoction was left in air for an hour to cool down. The decoction was collected and then extra 1:10 distilled water was

added to the raw materials to reflux for two more hours. Similarly, decoction was collected after one hour of cooling. The decoction was kept in 4℃ refrigerator overnight for further steps.

Filtration

Cold decoction was divided into several 450ml-centrifuge bottles and then centrifuged at 17,700 x *g* for an hour. Supernatant was collected and filtrated with filter paper under suction to filter out all the insoluble particles.

Lyophilization

Clear decoction was divided into lyophilize bottles, up to 300 ml each bottle. Bottles were frozen under liquid nitrogen and the frozen decoction was kept no more than one cm thick surrounding the inner side of the bottle. The bottles were dried on lyophilizer under vacuum for at least three days until dry powder appeared in the bottle. Powder extract was collected into falcon tubes and kept in a moisture proof box at room temperature.

2.2.2 Results

As shown in table 2.1, 98 g aqueous extract of Danshen (G), 22 g of Gegen (G), 9 g of Yanhu (G), 109 g of 6:3:1 (D:G:Y) compound formula, 40 g of 7:3 (D:G) compound formula, 39 g of 2:1 (D:G) compound formula and 35 g of 1:1 (D:G) compound formula were yielded from 180 g, 90 g, 30 g, 300 g, 100 g, 100 g and 100g raw materials respectively.

	Raw materials (g)	Powder extract (g)	Percentage yield (%)
Danshen (D)	180	97.557	54.20
Gegen (G)	90	21.645	24.05
Yanhu (Y)	30	9.087	30.29
6 : 3 : 1 (D:G:Y)	300	108.907	36.30
7 : 3 (D:G)	100	39.736	39.74
2 : 1 (D:G)	100	39.489	39.49
1 : 1 (D:G)	100	34.808	34.81

Table 2.1. The weights of raw material used and the amount of powder extract obtained. The percentage yield that calculated by weights shows individual Danshen gave the highest percentage yield while individual Gegen gave the lowest percentage yield.

2.2.3 Discussion

The percentage yield of Danshen (D), Gegen (G), Yanhu (Y), 6:3:1 (D:G:Y) compound formula, 7:3 (D:G) compound formula, 2:1 (D:G) compound formula and 1:1 (D:G) compound formula were found to be 54.20%, 24.05%, 30.29%, 36.30%, 39.74%, 39.49% and 34.81% respectively. The highest percentage yield was obtained from the extraction with Danshen while the one of Gegen was the lowest. Therefore a higher percentage yield was obtained from the mixture with a higher Danshen composition compound formula.

The extracts of compound formulae were prepared by mixing TCMs before decoction but not mixing the extract of individual TCM because there may be new compounds formed during the step of preparation of decoction. All the compounds could be obtained by this mixed TCMs decoction method when compared to the traditional decoction of the compound formula.

2.3 Preliminary test

An inhibition against 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) induced red blood cell hemolysis experiment was performed to compare the antioxidant ability of the focused compound formulae. AAPH was employed to produce peroxyl radicals, and erythrocyte would be lysed by lipid peroxidation. Therefore the antioxidant potency of the formulae could be compared by the amount of red blood cell lysed.

2.3.1 Material and methods

Animals

300-320 g Sprague-Dawley (SD) rat was treated with ether and then blood was collected by tubes containing heparin at the aorta. Red blood cells were obtained by centrifugation and they were washed twice with 0.1 M NaCl solution. The volume of red blood cell was measured and 4 times volume of 0.1 M NaCl solution was added to obtain a 20% red blood cell suspension.

Lysis reaction

Seven extracts from different formulae were weighed and dissolved in PBS and serial dilutions were performed to obtain a series of extracts in various concentrations. Reactions were set up in 1.5-ml micro-centrifuge tubes, each contained a final volume of 1 ml of 10% red blood cell suspension, 100 mM AAPH, and extracts. Final concentrations of the samples were 500 μg/ml, 250 μg/ml, 125 μg/ml, 62.5 μg/ml and 31.25 μg/ml. Controls were set up by using PBS instead of sample. Tubes were

incubated at 37℃ for 200 min with oscillation.

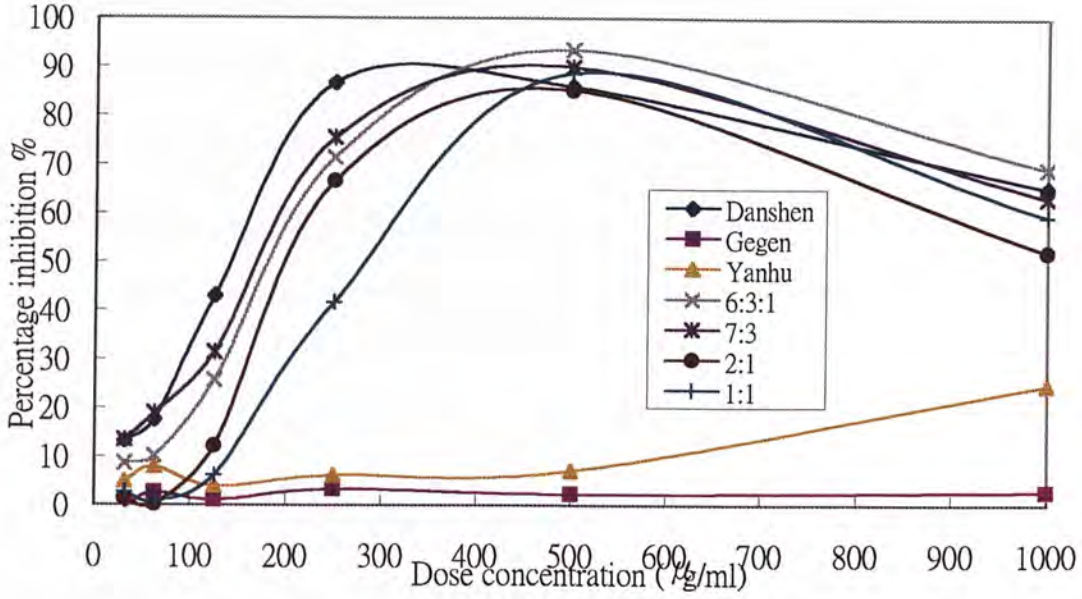
Determination of cell lysis

The incubated reaction mixtures were diluted 20 fold in PBS and distilled water separately. They were shaken gently and then centrifuged at 1500 x *g* for 10 min. Two hundred μl of supernatant were transferred to flat bottom 96-well plates and the absorbances of them were read against 540 nm. Percentage hemolysis was the absorbance of the sample in PBS over absorbance of the sample in distilled water times 100 percentage. The percentage inhibition was the percentage hemolysis of control minus the percentage hemolysis of sample. The 50% inhibition concentrations (IC50) were used to compare the potency of the formulae.

2.3.2 Results

The percentage inhibition of red blood cell hemolysis was plotted against the dose concentration of the seven samples, three individual TCMs and four compound formulae. As shown in figure 2.1, Gegen showed no antioxidant effect against AAPH and Yanhu showed little effect, 25% inhibition up to 1 mg/ml dosage. IC50 of Gegen and Yanhu could not be detected within 1 mg/ml dose range. IC50 of Danshen, compound formulae 6:3:1 (D:G:Y), 7:3(D:G), 2:1 (D:G) and 1:1 (D:G) were 141 μg/ml, 180 μg/ml, 168 μg/ml, 198 μg/ml and 282 μg/ml respectively.

a)



b)

Conc. (μg/ml)	Percentage inhibition %						
	Danshen	Gegen	Yanhu	6:3:1 (D:G:Y)	7:3 (D:G)	2:1 (D:G)	1:1 (D:G)
1000	65.12 ± 10.69	2.64 ± 2.16	24.47 ± 14.90	69.14 ± 10.75	63.08 ± 14.83	51.96 ± 10.67	59.31 ± 7.19
500	85.96 ± 6.05	2.24 ± 4.09	7.06 ± 5.87	93.54 ± 3.80	90.02 ± 5.11	85.16 ± 6.35	88.68 ± 3.90
250	86.70 ± 2.89	3.24 ± 4.31	6.13 ± 9.12	71.41 ± 8.85	75.43 ± 10.12	66.55 ± 13.13	41.66 ± 13.53
125	42.98 ± 9.33	1.03 ± 5.07	3.86 ± 4.98	25.65 ± 12.35	31.42 ± 17.87	12.17 ± 13.40	6.14 ± 7.88
62.5	17.56 ± 7.61	2.59 ± 5.83	7.81 ± 6.16	10.18 ± 7.88	18.89 ± 10.83	0.22 ± 2.75	0.87 ± 5.35
31.25	13.26 ± 6.04	-0.41 ± 3.19	4.86 ± 7.58	8.70 ± 5.47	13.44 ± 15.67	1.37 ± 2.53	2.68 ± 6.74

Figure 2.1. Percentage inhibition of the 3 individual formulae, Danshen (D), Gegen (G) and Yanhu (Y) and the 4 compound formulae, 6:3:1 (D:G:Y), 7:3 (D:G), 2:1 (D:G) and 1:1 (D:G). IC50 were 141 μg/ml, undetectable, undetectable, 180 μg/ml, 168 μg/ml, 198 μg/ml and 282 μg/ml respectively. a) Curves are plotted by mean of n=7. b) Data are tabulated by mean ± SD of n=7.

2.3.3 Discussion

Danshen alone gave the lowest IC50 implying that Danshen was most effective in antioxidant activity against AAPH-induced RBC hemolysis. For the compound formulae, the higher the Danshen composition gave the lower IC50. Therefore the higher Danshen composition gave the higher antioxidant effect. The 7:3 (D:G) compound formula was the one showing the highest Danshen composition among the four formulae.

In addition, individual Yanhu only showed a 25% inhibition up to the dose of 1000 μg/ml. Its traditional application is not related to cardiovascular tonic effect but only to analgesic effect. However, according to the physicians views, the analgesic effect of Yanhu may mask the clinical observation after the extract is capsulated and used for clinical trial because it is dangerous if atients are suffering from ischemia-induced pain but the painful sign is alleviated by Yanhu. Therefore Yanhu was eliminated from the compound formula and the finalized target ratio is 7:3 of Danshen to Gegen for further studies in this thesis.

Chapter 3 Quality Control

Quality of natural health products, and quality of herbal products in particular, has stood out as one of the most frequently cited critical issues in commercial world. Quality control for TCM products is not only the chemical content of the products itself, but also a broad regulatory perspective including authentication to avoid adulterants and substitutes, chemical content of the marker compounds, biological activity of the efficacy of product.

Marker compounds are one or more constituents that occur naturally in the herbal material and that are selected for special attention by a researcher or manufacturer as a marker for quality control purpose (Ho and Zheng, 2002). The selection may be based upon a variety of different factors such as the stability of the constituent, technical ease of analysis, amount of time and cost of analysis, utility in confirming identification of the component herbals, potential relevance to therapeutic effect and indicator of product quality.

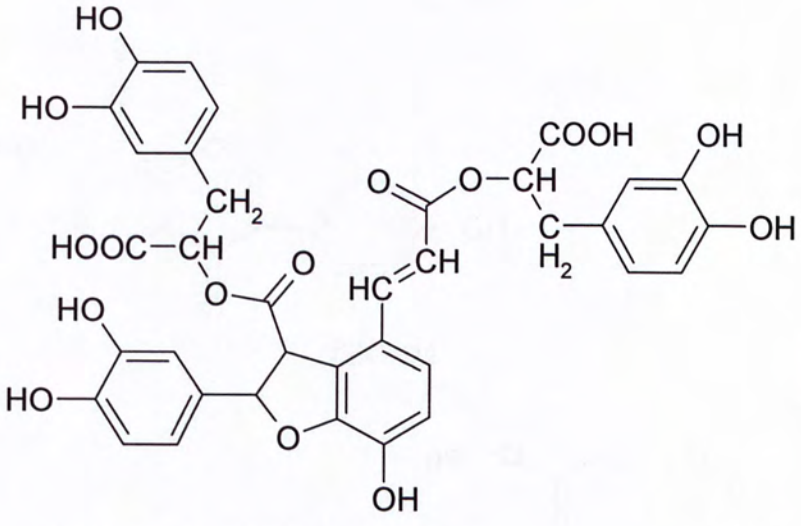
The chemical content quality of the 7:3 (D:G) compound formula was investigated in this section by applying the high-performance liquid chromatography (HPLC) method.

3.1 HPLC standardization

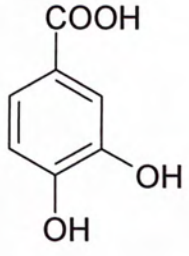
There are a number of active constituents which were identified from Danshen, such as the lipid-soluble compounds tanshinones I, tanshinones IIA, tanshinones IIB, water-soluble compounds protocatechualdehyde, salvianolic acid B and lithospermic acid as shown in figure 3.1. There are also a number of active constituents which were identified from Gegen. Most of them are isoflavones or their derivatives, such as puerarin, daidzin, daidzein and genistein as shown in figure 3.2.

For the quality control purpose, Puerarin was chosen to be the marker compound of Gegen. Protocatechualdehyde and Salvianolic acid B were chosen to be the marker compounds of Danshen.

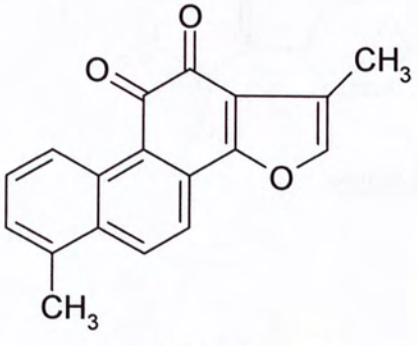
Puerarin is the most abundant active constituent in Gegen. Formula weight of Puerarin is 422.3 and can be detected under UV 250nm. Protocatechualdehyde and Salvianolic acid B are active constituents in the aqueous fraction of Danshen. Formula weights of them are 138.1 and 718.0 respectively, and both of Protocatechualdehyde and Salvianolic acid B can be detected under UV 280nm.



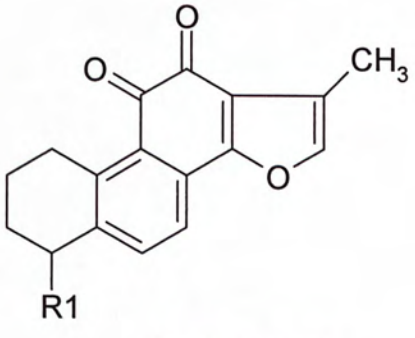
Salvianolic acid B



Protocatechualdehyde

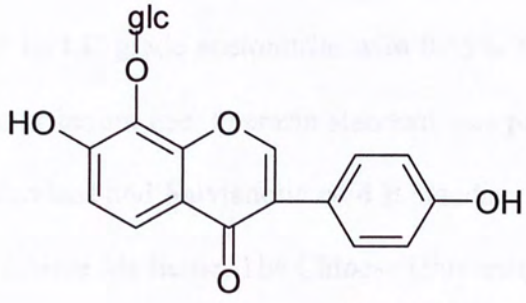


Tanshinone I

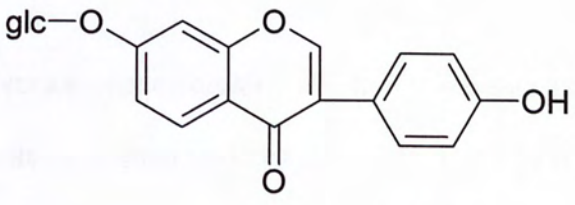


Tanshinone II
A: R1 = CH_3
B: R1 = CH_2OH

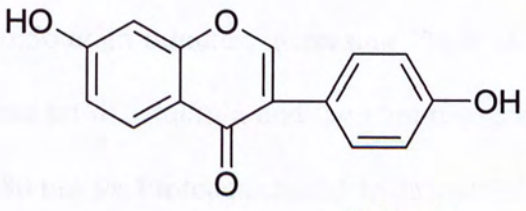
Figure 3.1. Structures of some active constituents of Danshen.



Puerarin



Daidzin



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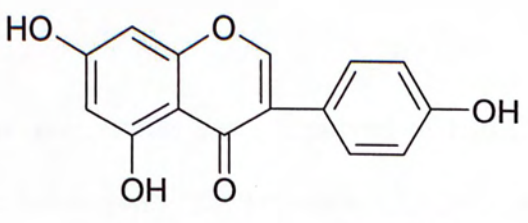


Genistein

Figure 3.2. Structures of some active constituents of Gegen. Where glc represent glucose.

3.2 Materials and methods

Chemicals

Solvent A is HPLC grade water with 0.05% trifluoroacetic acid (TFA). Solvent B is HPLC grade acetonitrile with 0.05% TFA. Solvent A and B were degassed for an hour before use. Puerarin standard was purchased from Sigma. Protocatechualdehyde standard and Salvianolic acid B standard were purified and supplied by Institution of Chinese Medicine, The Chinese University of Hong Kong.

HPLC conditions

An octadecyl silica (ODS) reversed phase column (150 mm x 4.6 mm i.d.) was employed using a linear gradient solvent system (solvent A:B from 100:0 at 0 min to 50:50 at 25 minutes, increasing 2% B per minute) as the mobile phase. The flow rate was set at 1 ml/min and the chromatogram was detected with an UV-detector at UV 280 nm for Protocatechualdehyde and Salvianolic acids B, and 250 nm for Puerarin.

Sample preparation

The extracts and standard marker compounds were dissolved in HPLC grade water. They were sonicated for 30 min and were passed through a 0.22 μm spore size filter before injecting to the HPLC system.

3.3 Results

The HPLC chromatographs of standards namely, Protocatechualdehyde, Salvianolic acid B and Puerarin were shown in figures 3.3, 3.4 and 3.5 respectively. Areas of 9276.77 mAUs, 13549.8 mAUs and 8153.42 mAUs were yielded by 2 μg Protocatechualdehyde, 15 μg Salvianolic acid B and 1.6 μg Puerarin with retention time 9.560 min, 17.555 min and 11.872 min respectively. The ratios of the area to the amount of marker compounds were used for further calculations.

The HPLC chromatograph of 100 μg Danshen aqueous extract was shown in figure 3.6. By comparing the retention time to the marker standards, the areas of peaks of Protocatechualdehyde and Salvianolic acid B were 537.18 mAUs and 5365.61 mAUs respectively. Therefore, the Protocatechualdehyde and Salvianolic acid B contents in Danshen extract were 0.12 μg and 5.94 μg respectively. The HPLC chromatograph of 100 μg Gegen aqueous extract was shown in figure 3.7. By comparing the retention time to the marker standards, the area of peak of Puerarin was 3693.83 mAUs. Therefore, the Puerarin content in Gegen extract was 0.72 μg.

The HPLC chromatographs of 100 μg 7:3 (D:G) compound formula were shown in figures 3.8 and 3.9. By comparing the retention time to the marker standards, the areas of peaks of Protocatechualdehyde, Salvianolic acid B and Puerarin were 307.99 mAUs, 5910.02 mAUs and 492.80 mAUs respectively. Therefore, the Protocatechualdehyde, Salvianolic acid B and Puerarin contents in 7:3 (D:G) compound formula were 0.07 μg, 6.54 μg and 0.10 μg respectively.

Figure 3.3. HPLC chromatogram (280 nm) of Protocatechualdehyde standard. Ten μl of 0.2 mg/ml Protocatechualdehyde was injected. The peak of Protocatechualdehyde was shown at retention time 9.560 min. Area under the peak was calculated to be 9276.77 mAUs.

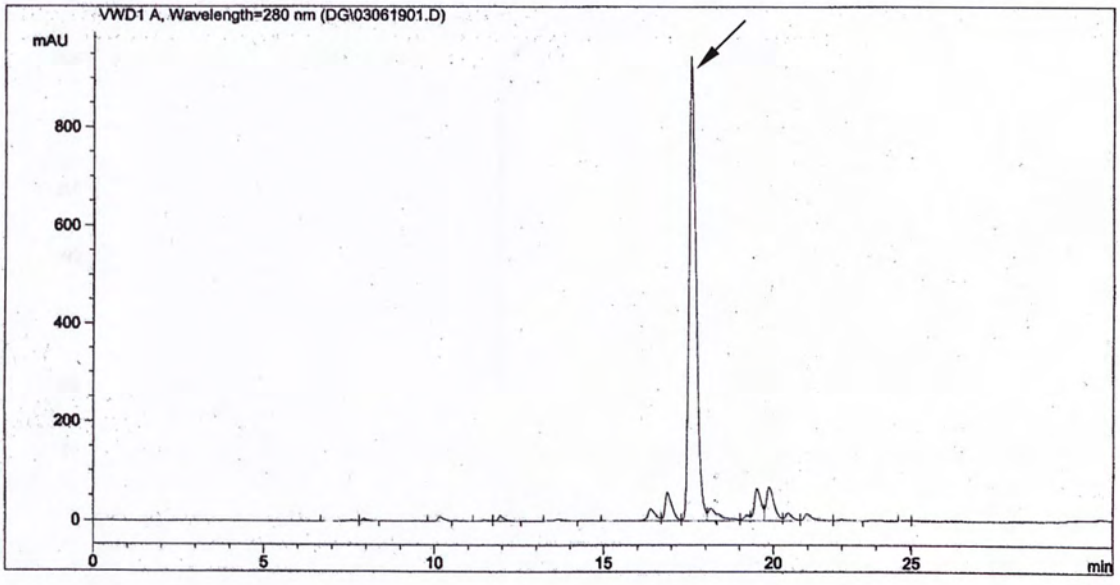


Figure 3.4. HPLC chromatogram (280 nm) of Salvianolic acid B standard. Ten μl of 0.2 mg/ml Salvianolic acid B was injected. The peak of Salvianolic acid B was shown at retention time 17.555 min. Area under the peak was calculated to be 13549.8 mAUs.

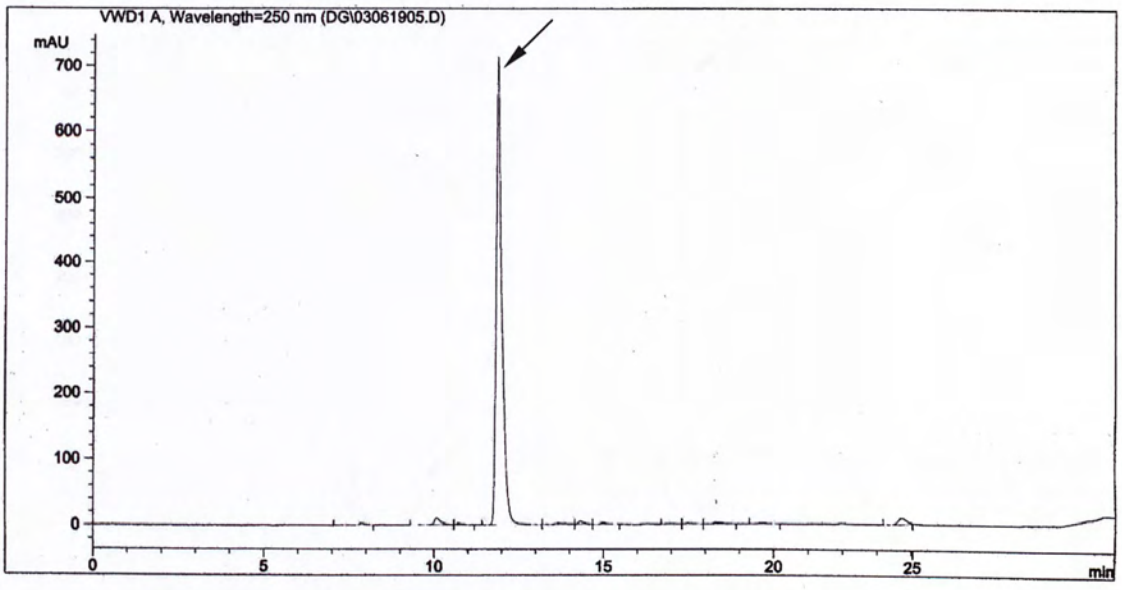


Figure 3.5. HPLC chromatogram (250 nm) of Puerarin standard. Ten µl of 0.16 mg/ml puerarin was injected. The peak of Puerarin was shown at retention time 11.872 min. Area under the peak was calculated to be 8153.42 mAUs.

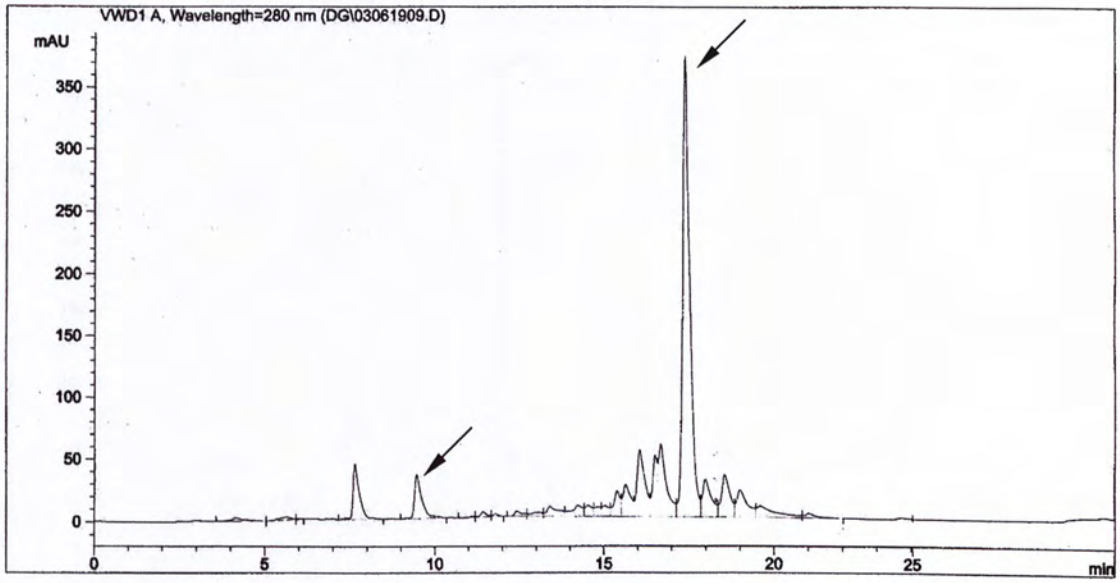


Figure 3.6. HPLC chromatogram (280 nm) of Danshen aqueous extract. Fifty μl of 2 mg/ml Danshen aqueous extract was injected. The peak shown at retention time 9.467 min should be Protocatechualdehyde and the area under the peak was calculated to be 537.18 mAUs. The peak shown at retention time 17.374 min should be Salvianolic acid B and the area under the peak was calculated to be 5365.61 mAUs.

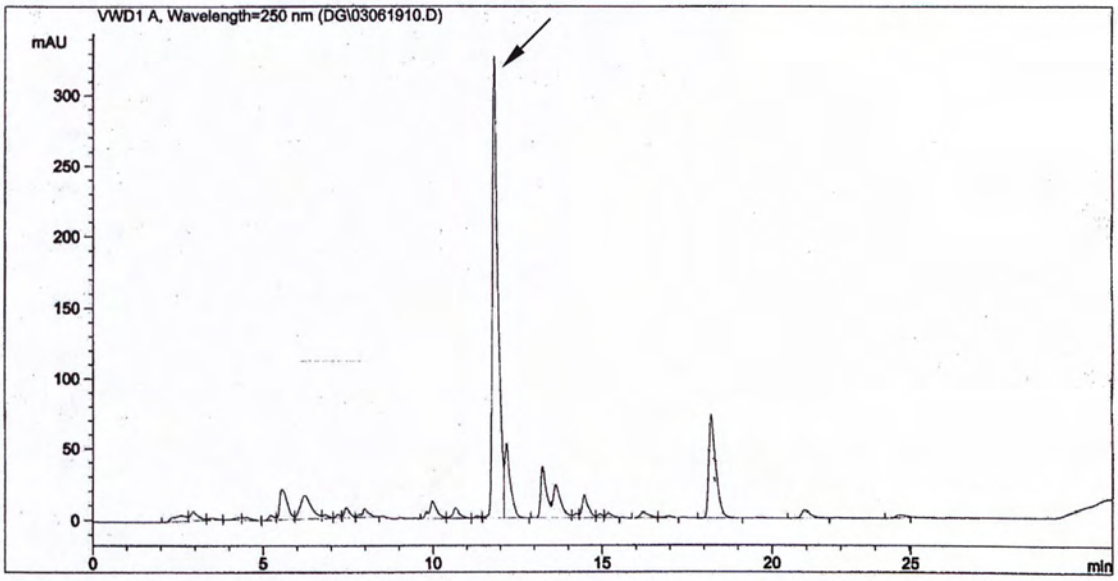


Figure 3.7. HPLC chromatogram (250 nm) of Gegen aqueous extract. Fifity μl of 2 mg/ml Gegen aqueous extract was injected. The peak shown at retention time 11.817 min should be Puerarin and the area under the peak was calculated to be 3693.83 mAUs.

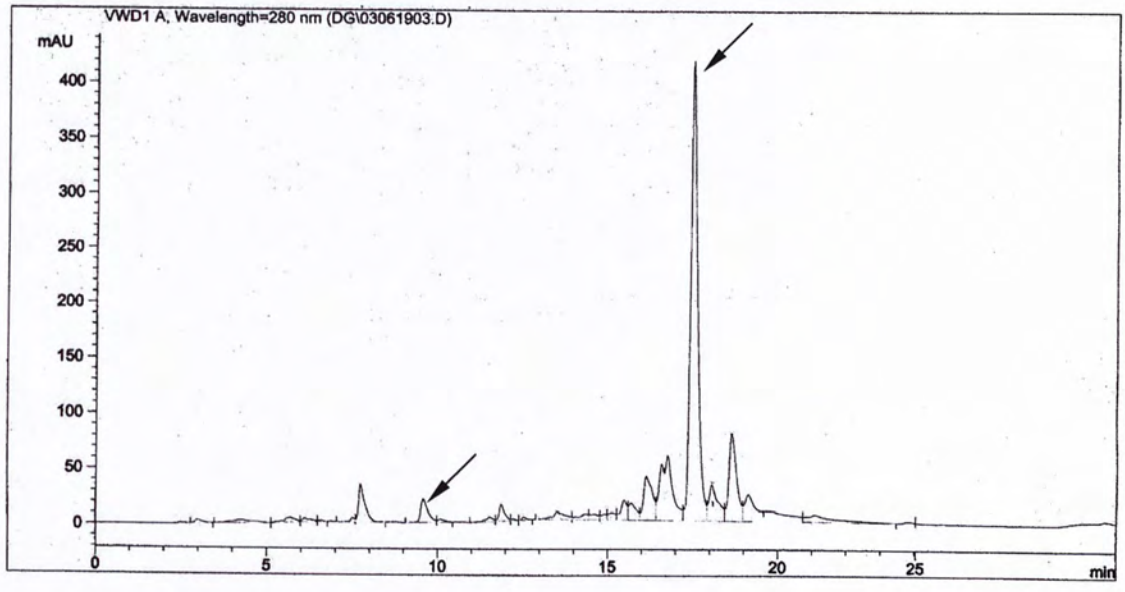


Figure 3.8. HPLC chromatogram (280 nm) of 7:3 (D:G) compound formula. Fifity μl of 2 mg/ml 7:3 (D:G) compound formula was injected. The peak shown at retention time 9.569 min should be Protocatechualdehyde and the area under the peak was calculated to be 307.99 mAUs. The peak shown at retention time 17.463 min should be Salvianolic acid B and the area under the peak was calculated to be 5910.02 mAUs.

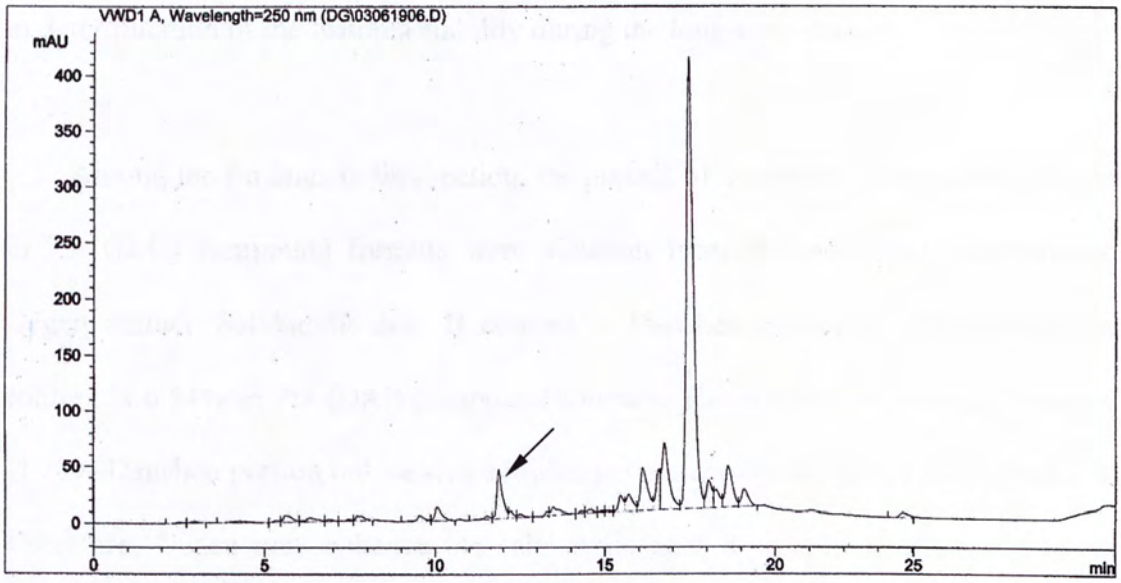


Figure 3.9. HPLC chromatogram (250 nm) of 7:3 (D:G) compound formula. Fifity µl of 2 mg/ml 7:3 (D:G) compound formula was injected. The peak shown at retention time 11.864 min should be Puerarin and the area under the peak was calculated to be 492.80 mAUs.

3.4 Discussion

By applying the HPLC method, the Protocatechualdehyde, Salvianolic acid B and Puerarin contents in 7:3 (D:G) compound formula were 0.07% , 6.54% and 0.10% by weight respectively. These data are useful for monitoring the batch to batch quality of the formula production. The contents of marker compounds are also useful in determination of the formula stability during the long-term storage.

Among the findings in this section, the portion of the marker compounds yielded in 7:3 (D:G) compound formula were different from the individual Danshen and Gegen extract. Salvianolic acid B content in Danshen extract is 5.94% while the content is 6.54% in 7:3 (D:G) compound formula. The compound formula consisted of 70% Danshen portion but yielded a higher percentage content of salvianolic acid B. Therefore, Gegen may enhance the salvianolic acid B extraction efficiency by an unknown mechanism. It may explain the philosophy of TCM used in compound formula in a scientific point of view. As in Chinese medicine, the TCMs are always combined in appropriate ratio for decoction. And some TCMs are always used together to enhance one another or to neutralize one another in terms of synergistic effect.

Chapter 4 Antioxidant study

For long-term prevention of cardiovascular disease, decreasing the oxidative stress of the blood stream by antioxidant agents is effective. Attack with reactive radicals on membranes or lipoproteins starts lipid peroxidation, which is particularly implicated in the development of atherosclerosis (Halliwell, 1997). Atherosclerosis is one of the major chronic cardiovascular diseases. Atherosclerosis will narrow down the cross section area of the vessels, forming foams fatty streak first, and then fibrous plaque and finally becoming complicated lesion. The formation of atherosclerosis is related to the oxidative stress of the blood stream. Low density lipoprotein (LDL) is first oxidized by free radicals at the intima and they are then aggregated together. After that, they cause damage to the endothelium (Cochrane, 1991) and turn on the immune system. More macrophages will migrate to the site and inflammation occurrs. The scavenger receptors on the surface of macrophages and smooth muscle will interact with oxidized LDL to induce the formation of foam cells (Suematsu *et al.*, 1993 ; Ward, 1991). Foam cells cannot be easily removed and they are continuously accumulating with lipids and calcium to narrow down the vessels.

Two models were applied to investigate the antioxidant effect of the 7:3 (D:G) compound formula. The two models are AAPH induced red blood cell hemolysis and ischemia reperfusion of isolated rat heart on Langendorff apparatus.

4.1 Red blood cell hemolysis model

An inhibition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), induced red blood cell hemolysis experiment has been performed to examine the antioxidant ability of the compound formula at 7:3 (D:G).

4.1.1 Materials and Methods

Same as described in section 2.3.1.

4.1.2 Results

Sharing the results obtained in section 2.3.2, the data on the percentage inhibition of red blood cell hemolysis were re-plotted against the dose concentration of the three samples, Danshen (D) and Gegen (G) alone, and the 7:3 (D:G) compound formula as shown in figure 4.1. Gegen showed no antioxidant effect against AAPH. IC50 of Gegen could not be detected within 1 mg/ml dose range. IC50 of Danshen and compound formula 7:3 (D:G) were found to be 141 μg/ml and 168 μg/ml respectively.

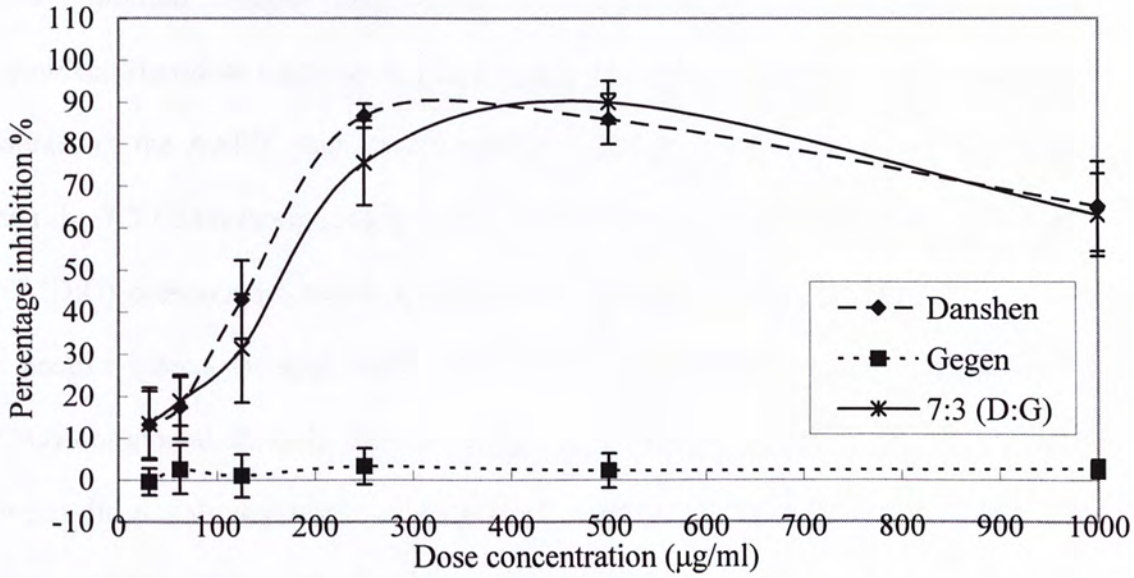


Figure 4.1. Percentage inhibition of AAPH induced hemolysis of the individual Danshen (D) and Gegen (G), and the 7:3 (D:G) compound formula. IC50 were 141 μg/ml, undectectable and 168 μg/ml respectively. Data are represented by mean ± SD of n=7.

4.1.3 Discussion

Percentage hemolysis was the absorbance of the sample in PBS over absorbance of the sample in distilled water times 100 percentage. The percentage inhibition was the percentage hemolysis of control minus the percentage hemolysis of sample. The 50% inhibition concentrations (IC50) were used to compare the potency of the formulae. Therefore the lower the IC50 value the higher the potency of the sample to counteract the AAPH induced red blood cell hemolysis. Danshen was more potent than the 7:3 (D:G) compound formula, as the IC50 of Danshen was 141 μg/ml while 7:3 (D:G) compound formula is 168 μg/ml. Although Gegen did not show positive protective effects, at least, with unknown mechanism, the protective effect of 7:3 (D:G) compound formula did not show a sudden drop in the presence of Gegen. Gegen did not show positive protection as an antioxidant did. However, it may play other role that affects other systems in the body to supplement the cardiovascular tonic actions.

The formation of atherosclerosis is related to the oxidative stress of the blood stream, e.g. decrease the chance of forming oxidizing LDL. Atherosclerosis may be prevented by daily supplement of 7:3 (D:G) compound formula as it can quench the oxidants. For the patients who are suffering atherosclerosis, 7:3 (D:G) compound formula may help as secondary prevention because the daily supplement of 7:3 (D:G) compound formula may maintain a high level of antioxidant that slows down the chance of atherosclerosis formation.

4.2 Ischemia-reperfusion on Langendroff

Ischemia is a situation that the oxygen supply is decreased that causes insufficient oxygen to carry out normal metabolism (Hearse, 1994). After ischemia, the restoration of oxygen supply is called reperfusion. Reperfusion restores the oxygen content but it will cause injury to the organ. It was because the level of hypoxanthine will be increased when ischemia occurs (Manning *et al.*, 1988). Hypoxanthine converses xanthine dehydrogenase into xanthine oxidase during ischemia (McCord *et al.*, 1985). Once the oxygen content is restored, the xanthine oxidase will turn oxygen into free radicals which damage cells and cause the ischemia-reperfusion injury.

To examine the effect of the extracts on the ischemia-reperfusion injury, an isolated rat heart was cannulated on a Langendorff apparatus and an *ex-vivo* ischemia-reperfusion injury assay was performed. Extracts of compound formula were added before reperfusion in order to investigate the protective effects against the ischemia-reperfusion injury. Four parameters were monitored to compare the heart function. The four parameters are heart contractile force, coronary flow rate, and the heart specific enzyme activities, namely, lactate dehydrogenase and creatine kinase activities.

4.2.1 Materials and Methods

Chemicals

Heparin sodium salt from porcine intestinal mucosa, creatine kinase diagnostic kit and lactate dehydrogenase diagnostic kit were purchased from Sigma. Krebs solution (118.0 mM NaCl, 4.7 mM KCl, 1.64 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.55 mM D-glucose, 25.0 mM $NaHCO_3$ and 2.5 mM $CaCl_2$) was adjusted to pH 7.4 and filtered.

Animals

220-250 g male Sprague-Dawley (SD) rat was killed by cervical dislocation. The chest was opened and the heart was excised within one minute. The excised heart was immediately put into cold heparinized Krebs solution with 50 unit/ml heparin to prevent blood clotting. The surrounding connective tissues and fats were quickly removed and therefore a single opening aorta was recognized. The excised heart was cannulated on a Langendroff apparatus.

Ischemia-Reperfusion on Langendorff apparatus

The aorta of rat heart was cannulated on the Langendorff apparatus and held tightly by a wire. Thirty-seven ℃ pre-warmed and 95% oxygen with 5% carbon dioxide gassed Krebs solution was allowed to perfuse into the heart. A basal level force was applied to the rat heart to induce its contraction. The force was increased up to 2 g at the increasing rate less than 0.5 g per minute. The rat heart was left for 20 minutes for equilibration. The supply of Krebs solution was stopped and started ischemia for 29 minutes. Two hundred μl of sample was injected to the reservoir

before a 15-minute length reperfusion. Krebs solution was used instead of the sample for the control set. Measurements with 17 time points were made at 10 min and 20 min during the equilibration period, and every minute during the 15-minute reperfusion period.

Contractile force measurement

The contractile force was determined by a wire which was connected to a force-displacement transducer. The force displacement transducer was connected to a Polygraph to plot out the contractile force. The percentage contractile force recovery was the contractile force during reperfusion compared to the average values at 10^{th} minute and 20^{th} minute equilibration period of the same rat.

Coronary flow rate measurement

Amount of the coronary heart filtrate was collected and measured for one minute. By making an assumption that density of the heart filtrate is 1 g/ml, same as water, the weight of the heart filtrate collected within the particular one minute is the average flow rate in the units of ml/min. The percentage coronary flow rate recovery was the coronary flow rate during reperfusion compared to the average values at 10th minute and 20th minute of equilibration of the same rat.

Lactate dehydrogenase activity measurement

Fifity µl of the heart filtrate was added to a pre-warmed 30°C cuvette containing 1 ml lactate dehydrogenase reagent and mixed by inversion. After 30 seconds incubation, the absorbance was read at wavelength 340 nm versus water as reference. Absorbances were read at 30-second intervals for a period of 60 seconds. The activity

of lactate dehydrogenase in the unit of U/L is the change of absorbance per minute (ΔA/min) times 3376.

Creatine kinase activity measurement

Twenty μl of the heart filtrate was added to a pre-warmed 30℃ cuvette containing 1 ml creatine kinase reagent and mixed by inversion. After three minutes incubation, the absorbance was read at wavelength 340 nm versus water as reference. Absorbances were read at 30-second intervals for a period of 120 seconds. The activity of creatine kinase in the unit of U/L is the change of absorbance per minute (ΔA/min) times 8200.

4.2.2 Results

Dose responses on ischemia-reperfusion protection of the three samples, 7:3 (D:G) compound formulae, individual Danshen (D) and Gegen (G), were investigated. Five concentrations, 1 mg/ml, 2 mg/ml, 4 mg/ml, 8mg/ml and 16 mg/ml, of the three samples were examined.

a) 7:3 (D:G) compound formula

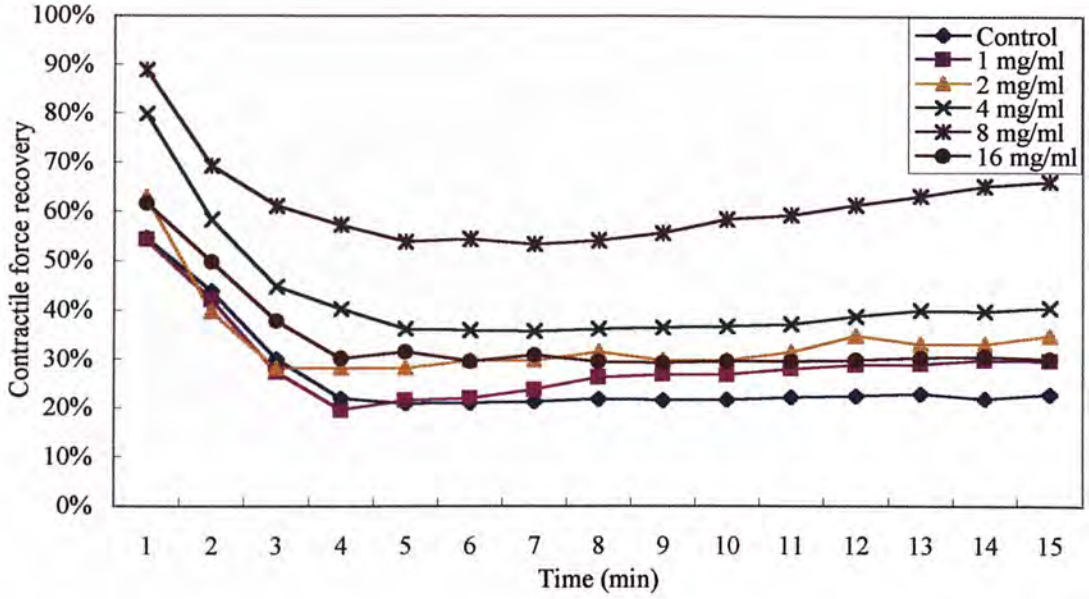
Protective effect of 7:3 (D:G) compound formula was demonstrated at contractile force recovery during reperfusion. As shown in figure 4.2, dose from 1 mg/ml to 4 mg/ml showed a small protection at around 30% recovery when compared to the control at about 20% recovery. Obvious protection was shown when the dose increased to 8 mg/ml, where the recovery was around 60%. But the protective effect was reduced when the dose was increased up to 16 mg/ml, where the recovery dropped to 30%.

Protective effect of 7:3 (D:G) compound formula was also demonstrated at coronary flow rate recovery during reperfusion when the dose is 2 mg/ml or higher. As shown in figure 4.3, dose of 1 mg/ml did not show protection at around 40% recovery, as the same as the control. Dose of 2 mg/ml showed around 60% recovery and dose of 4 mg/ml showed around 80% recovery. Obvious protection was shown when the dose was increased to 8 mg/ml, where the recovery was over 100% at some time points. But the protective effect was reduced when the dose was increased up to 16 mg/ml, where the flow rate recovery dropped to around 65%.

Protective effect of 7:3 (D:G) compound formula was demonstrated at accumulating leakage of lactate dehydrogenase during reperfusion when the dose between 2 mg/ml to 8 mg/ml were used. As shown in figure 4.4, dose of 1 mg/ml did not show protection that 4.8 U of lactate dehydrogenase which was similar to the control of 4.5 U. When the dose was increased from 2 mg/ml to 8 mg/ml, the amount of lactate dehydrogenase was reduced from 3.9 U to 3.3 U. But the protection effect was reduced when the dose was increased up to 16 mg/ml, where the amount of lactate dehydrogenase was 5.3 U.

Protective effect of 7:3 (D:G) compound formula was demonstrated at accumulating leakage of creatine kinase during reperfusion when the dose between 2 mg/ml to 8 mg/ml were used. As shown in figure 4.5, dose of 1 mg/ml did not show protection that 13.6 U of creatine kinase was leaked out, similar to the control of 13.6 U. When the dose was increased from 2 mg/ml to 4 mg/ml, the amount of creatine kinase was reduced from 11.9 U to 10.7 U. Obvious protection was shown when the dose was increased to 8 mg/ml, where the amount of creatine kinase dropped to 6.2 U. But the protection effect was reduced when the dose was increased up to 16 mg/ml, where the amount of creatine kinase was 16.0 U that was much more than the control.

a)

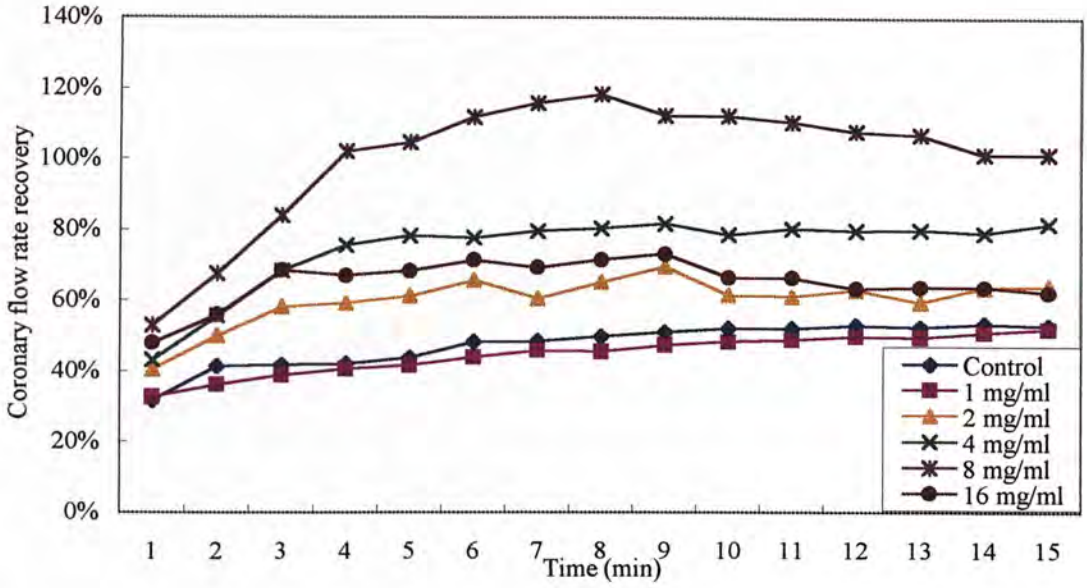


b)

Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	55% ± 18%	54% ± 15%	63% ± 1%	80% ± 33%	89% ± 7%	62% ± 27%
2	44% ± 17%	42% ± 12%	40% ± 8%	58% ± 6%	69% ± 27%	50% ± 18%
3	30% ± 11%	27% ± 5%	28% ± 10%	45% ± 10%	61% ± 24%	38% ± 25%
4	22% ± 8%	19% ± 10%	28% ± 9%	40% ± 9%	57% ± 21%	30% ± 19%
5	21% ± 7%	21% ± 11%	28% ± 7%	36% ± 13%	54% ± 19%	31% ± 20%
6	21% ± 6%	22% ± 10%	30% ± 4%	36% ± 5%	54% ± 20%	30% ± 20%
7	21% ± 6%	24% ± 8%	30% ± 5%	36% ± 4%	53% ± 16%	31% ± 22%
8	22% ± 5%	26% ± 6%	31% ± 6%	36% ± 8%	54% ± 16%	29% ± 22%
9	22% ± 6%	27% ± 4%	30% ± 5%	36% ± 7%	56% ± 16%	29% ± 22%
10	22% ± 6%	27% ± 5%	30% ± 7%	37% ± 9%	58% ± 16%	30% ± 21%
11	22% ± 6%	28% ± 4%	31% ± 8%	37% ± 9%	59% ± 17%	30% ± 21%
12	22% ± 6%	29% ± 5%	35% ± 8%	39% ± 5%	61% ± 18%	30% ± 21%
13	23% ± 6%	29% ± 4%	33% ± 8%	40% ± 6%	63% ± 19%	30% ± 21%
14	22% ± 9%	30% ± 4%	33% ± 5%	40% ± 9%	65% ± 18%	31% ± 21%
15	23% ± 10%	30% ± 5%	35% ± 10%	41% ± 8%	66% ± 18%	30% ± 22%

Figure 4.2. Contractile force recovery of ischemia-reperfusion injured heart protected by 7:3 (D:G) compound formula at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)

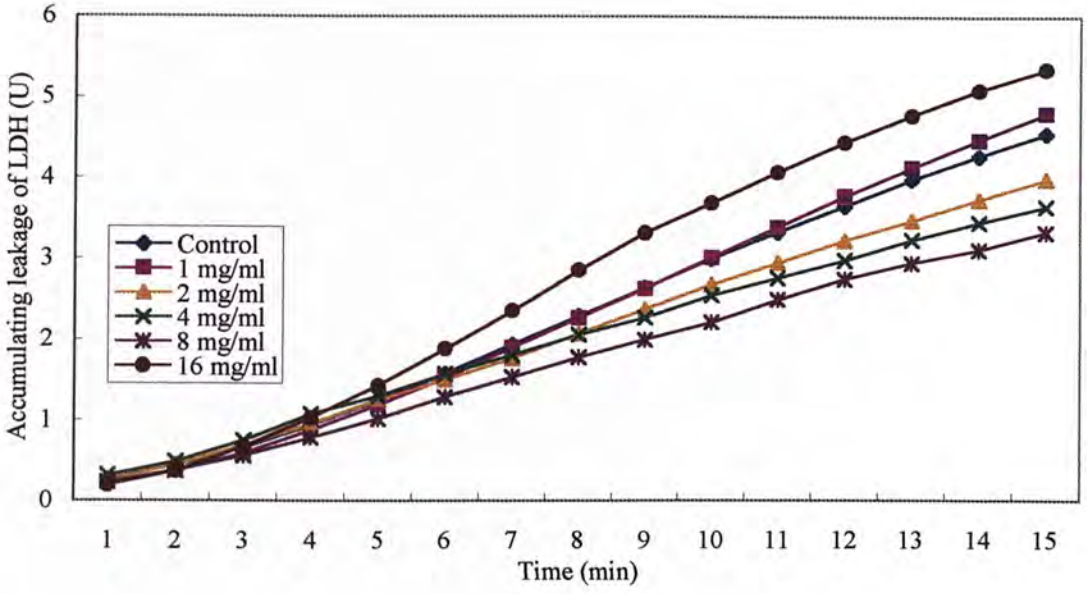


b)

Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	32% ± 8%	33% ± 7%	40% ± 12%	43% ± 11%	53% ± 27%	48% ± 27%
2	41% ± 8%	36% ± 4%	50% ± 12%	56% ± 0%	67% ± 25%	56% ± 20%
3	42% ± 8%	39% ± 5%	58% ± 12%	68% ± 5%	84% ± 23%	68% ± 35%
4	42% ± 8%	41% ± 6%	59% ± 15%	76% ± 7%	102% ± 21%	67% ± 34%
5	44% ± 8%	42% ± 5%	61% ± 13%	78% ± 9%	105% ± 27%	68% ± 35%
6	48% ± 8%	44% ± 7%	66% ± 13%	78% ± 9%	112% ± 32%	71% ± 34%
7	49% ± 8%	46% ± 8%	60% ± 10%	80% ± 9%	116% ± 30%	69% ± 35%
8	50% ± 8%	46% ± 8%	65% ± 12%	80% ± 11%	118% ± 32%	71% ± 32%
9	51% ± 8%	47% ± 8%	70% ± 10%	82% ± 14%	112% ± 28%	73% ± 30%
10	52% ± 8%	48% ± 9%	61% ± 10%	79% ± 11%	112% ± 26%	66% ± 32%
11	52% ± 8%	49% ± 10%	61% ± 9%	80% ± 14%	111% ± 25%	66% ± 29%
12	53% ± 8%	50% ± 10%	63% ± 9%	80% ± 16%	108% ± 22%	63% ± 30%
13	53% ± 8%	50% ± 11%	59% ± 8%	80% ± 14%	107% ± 20%	64% ± 28%
14	54% ± 8%	51% ± 12%	64% ± 10%	79% ± 14%	101% ± 20%	64% ± 26%
15	53% ± 8%	52% ± 13%	64% ± 13%	82% ± 13%	102% ± 16%	62% ± 24%

Figure 4.3. Coronary flow rate recovery of ischemia-reperfusion injured heart protected by 7:3 (D:G) compound formula at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)

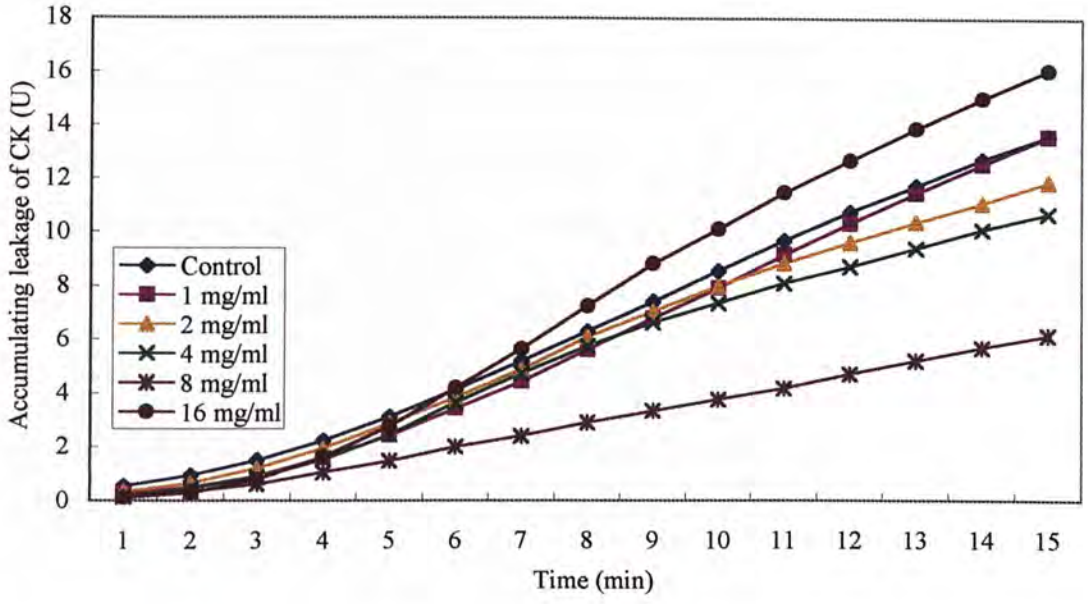


b)

	Accumulating leakage of LDH (U)					
Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	0.26 ± 0.12	0.22 ± 0.05	0.25 ± 0.06	0.31 ± 0.09	0.23 ± 0.09	0.19 ± 0.09
2	0.44 ± 0.13	0.37 ± 0.06	0.43 ± 0.08	0.49 ± 0.12	0.37 ± 0.11	0.37 ± 0.14
3	0.65 ± 0.14	0.58 ± 0.10	0.68 ± 0.10	0.74 ± 0.18	0.55 ± 0.15	0.67 ± 0.21
4	0.93 ± 0.16	0.87 ± 0.17	0.95 ± 0.12	1.08 ± 0.20	0.78 ± 0.18	1.02 ± 0.22
5	1.24 ± 0.18	1.18 ± 0.25	1.25 ± 0.14	1.30 ± 0.23	1.01 ± 0.20	1.43 ± 0.27
6	1.58 ± 0.21	1.52 ± 0.35	1.49 ± 0.18	1.57 ± 0.28	1.29 ± 0.26	1.88 ± 0.31
7	1.94 ± 0.23	1.90 ± 0.47	1.76 ± 0.20	1.81 ± 0.32	1.53 ± 0.25	2.35 ± 0.39
8	2.29 ± 0.28	2.26 ± 0.56	2.07 ± 0.22	2.06 ± 0.37	1.78 ± 0.25	2.85 ± 0.49
9	2.64 ± 0.35	2.63 ± 0.68	2.37 ± 0.25	2.27 ± 0.42	1.99 ± 0.25	3.31 ± 0.59
10	3.00 ± 0.43	3.01 ± 0.78	2.68 ± 0.27	2.54 ± 0.46	2.21 ± 0.27	3.68 ± 0.64
11	3.32 ± 0.53	3.38 ± 0.87	2.95 ± 0.31	2.76 ± 0.51	2.49 ± 0.34	4.06 ± 0.69
12	3.65 ± 0.63	3.76 ± 0.95	3.21 ± 0.35	2.98 ± 0.54	2.74 ± 0.40	4.44 ± 0.68
13	3.98 ± 0.69	4.12 ± 1.01	3.46 ± 0.36	3.23 ± 0.52	2.94 ± 0.37	4.77 ± 0.67
14	4.27 ± 0.77	4.47 ± 1.08	3.73 ± 0.39	3.44 ± 0.52	3.11 ± 0.38	5.09 ± 0.65
15	4.55 ± 0.80	4.80 ± 1.15	3.98 ± 0.43	3.64 ± 0.56	3.32 ± 0.43	5.35 ± 0.69

Figure 4.4. Accumulating leakage of lactate dehydrogenase of ischemia-reperfusion injured heart protected by 7:3 (D:G) compound formula at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)



b)

	Accumulating leakage of CK (U)					
Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	0.54± 0.29	0.26± 0.07	0.32± 0.05	0.13± 0.05	0.13± 0.05	0.11± 0.04
2	0.92± 0.34	0.48± 0.09	0.64± 0.10	0.45± 0.04	0.34± 0.09	0.28± 0.12
3	1.49± 0.37	0.89± 0.14	1.19± 0.15	0.85± 0.08	0.61± 0.16	0.77± 0.15
4	2.23± 0.43	1.58± 0.36	1.93± 0.25	1.53± 0.41	1.05± 0.26	1.62± 0.23
5	3.12± 0.56	2.42± 0.60	2.87± 0.50	2.45± 1.04	1.47± 0.37	2.77± 0.64
6	4.13± 0.74	3.42± 0.97	3.84± 0.82	3.65± 1.82	2.00± 0.43	4.20± 1.21
7	5.20± 0.97	4.44± 1.28	4.87± 1.24	4.75± 2.61	2.41± 0.60	5.65± 1.88
8	6.30± 1.23	5.61± 1.69	6.10± 1.70	5.76± 3.16	2.91± 0.73	7.25± 2.66
9	7.42± 1.54	6.80± 2.05	7.06± 2.14	6.65± 3.64	3.35± 0.89	8.83± 3.38
10	8.56± 1.91	7.91± 2.32	7.98± 2.43	7.37± 4.01	3.78± 1.02	10.11± 3.83
11	9.68± 2.37	9.15± 2.68	8.84± 2.72	8.10± 4.35	4.19± 1.15	11.48± 4.28
12	10.75± 2.74	10.32± 2.89	9.61± 2.89	8.72± 4.73	4.72± 1.21	12.68± 4.57
13	11.74± 3.08	11.46± 3.17	10.37± 3.14	9.41± 5.01	5.22± 1.36	13.88± 4.91
14	12.74± 3.42	12.52± 3.38	11.08± 3.33	10.09± 5.22	5.70± 1.43	15.00± 5.23
15	13.61± 3.70	13.59± 3.60	11.86± 3.50	10.69± 5.51	6.17± 1.57	16.06± 5.42

Figure 4.5. Accumulating leakage of creatine kinase of ischemia-reperfusion injured heart protected by 7:3 (D:G) compound formula at various dose. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

b) Danshen

Protective effect of Danshen was demonstrated at contractile force recovery during reperfusion. As shown in figure 4.6, the contractile force recovery of control was around 20%. Danshen exhibited protection to the ischemia-reperfusion injury when the dose was increased from 1 mg/ml to 8 mg/ml, the contractile fore recovery was enhanced from around 30% to around 60 %. But the protectiive effect was reduced when the dose was increased up to 16 mg/ml, where the recovery dropped to remain around 10%

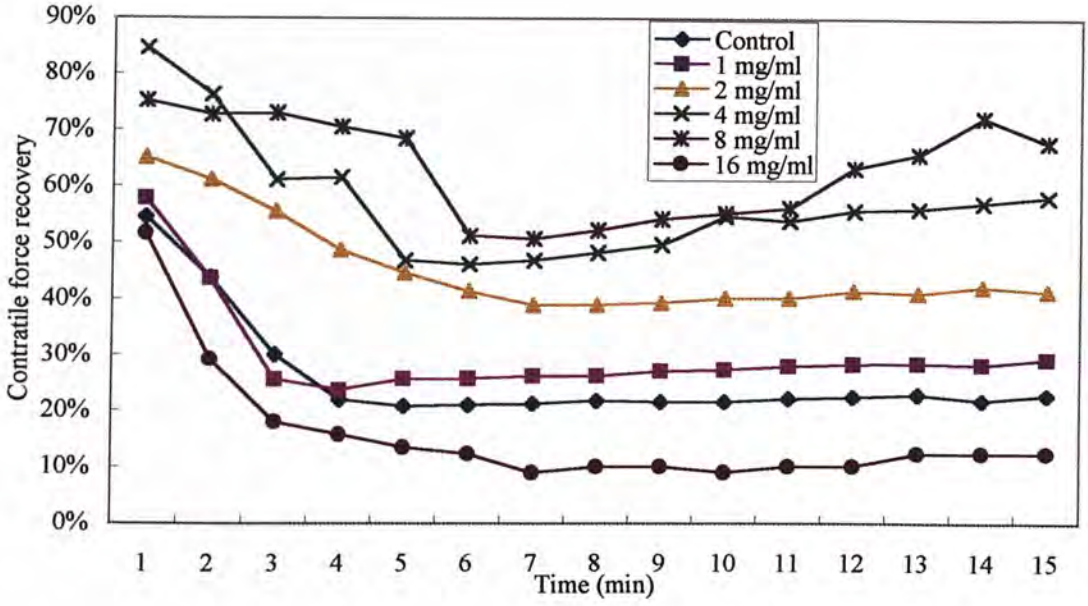
Protective effect of Danshen was demonstrated at coronary flow rate recovery during reperfusion when the dose is 2 mg/ml or higher. As show in figure 4.7, dose of 1 mg/ml did not show protection at around 40% to 50% recovery as the same as the control. Dose of 2 mg/ml showed around 80% recovery and dose of 4 mg/ml showed around 100% recovery. Obvious increased flow rate was shown when the dose was increased to 8 mg/ml, where the recovery was increased over 100% and reached 140%. But the protection effect was reduced when the dose was increased up to 16 mg/ml, where the flow rate recovery dropped to around 60%.

Protective effect of Danshen was demonstrated at accumulating leakage of lactate dehydrogenase during reperfusion when the dose between 4 mg/ml to 8 mg/ml were used. As shown in the figure 4.8, dose of 1 mg/ml and 2 mg/ml did not show much protection that 4.2 U and 3.9 U of lactate dehydrogenase respectively, which were slightly lower than the control of 4.5 U. When the dose was increased from 4 mg/ml to 8 mg/ml, the amount of lactate dehydrogenase was obviously dropped to 2.8

U and 2.2 U. But the protection effect was reduced when the dose was increased up to 16 mg/ml, where the amount of lactate dehydrogenase was 7.1 U that was much more than the control.

Protective effect of Danshen was demonstrated at accumulating leakage of creatine kinase during reperfusion when the dose between 1 mg/ml to 8 mg/ml were used. As shown in figure 4.9, dose of 1 mg/ml showed slightly protection that 12.4 U of creatine kinase was leaked when comparing to the control of 13.6 U. When the dose was increased from 2 mg/ml to 4 mg/ml, the amount of creatine kinase was reduced from 10.6 U to 9.8 U. Obvious protection was shown when the dose was increased to 8 mg/ml, where the amount of creatine kinase dropped to 4.1 U. But the protection effect was reduced when the dose was increased up to 16 mg/ml, where the amount of creatine kinase was 25.3 U that was much more than the control.

a)

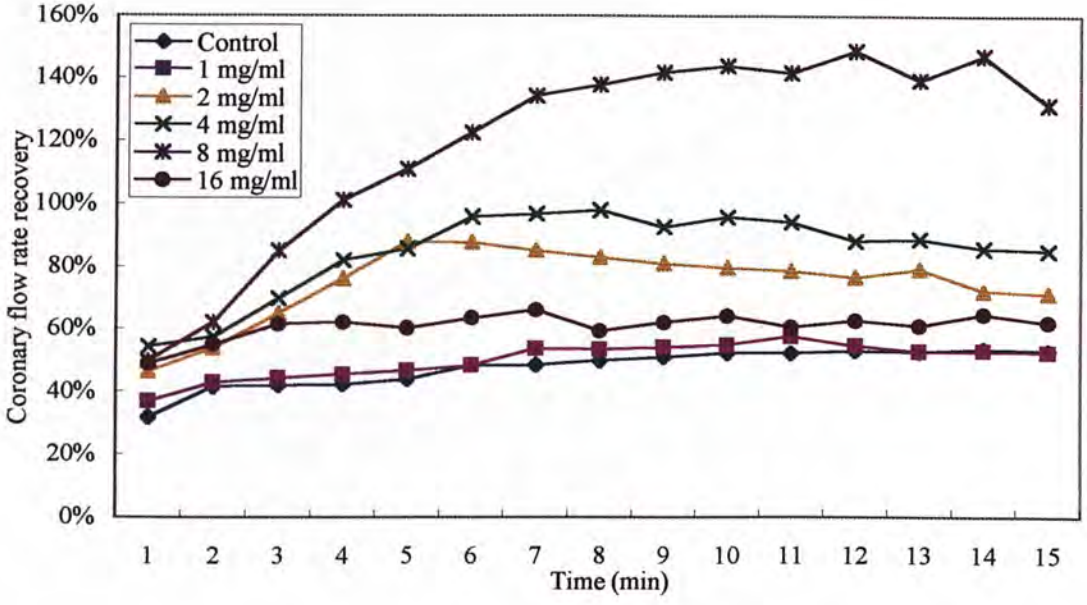


b)

Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	55% ± 18%	58% ± 19%	65% ± 29%	85% ± 16%	75% ± 19%	52% ± 20%
2	44% ± 17%	44% ± 24%	61% ± 34%	76% ± 30%	73% ± 22%	29% ± 12%
3	30% ± 11%	25% ± 13%	56% ± 36%	61% ± 29%	73% ± 20%	18% ± 8%
4	22% ± 8%	24% ± 10%	49% ± 35%	62% ± 31%	71% ± 17%	16% ± 5%
5	21% ± 7%	26% ± 8%	45% ± 31%	47% ± 20%	68% ± 10%	13% ± 9%
6	21% ± 6%	26% ± 7%	41% ± 28%	46% ± 19%	51% ± 26%	12% ± 8%
7	21% ± 6%	26% ± 7%	39% ± 24%	47% ± 20%	51% ± 25%	9% ± 5%
8	22% ± 5%	26% ± 7%	39% ± 23%	48% ± 21%	52% ± 27%	10% ± 5%
9	22% ± 6%	27% ± 8%	39% ± 24%	50% ± 22%	54% ± 29%	10% ± 4%
10	22% ± 6%	27% ± 8%	40% ± 24%	55% ± 21%	55% ± 30%	9% ± 8%
11	22% ± 6%	28% ± 9%	40% ± 25%	54% ± 25%	56% ± 31%	10% ± 6%
12	22% ± 6%	28% ± 8%	41% ± 24%	56% ± 26%	63% ± 24%	10% ± 6%
13	23% ± 6%	28% ± 9%	41% ± 25%	56% ± 27%	66% ± 27%	12% ± 7%
14	22% ± 9%	28% ± 8%	42% ± 25%	57% ± 28%	72% ± 21%	12% ± 4%
15	23% ± 10%	29% ± 10%	41% ± 26%	58% ± 29%	68% ± 29%	12% ± 6%

Figure 4.6. Contractile force recovery of ischemia-reperfusion injured heart protected by Danshen at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)

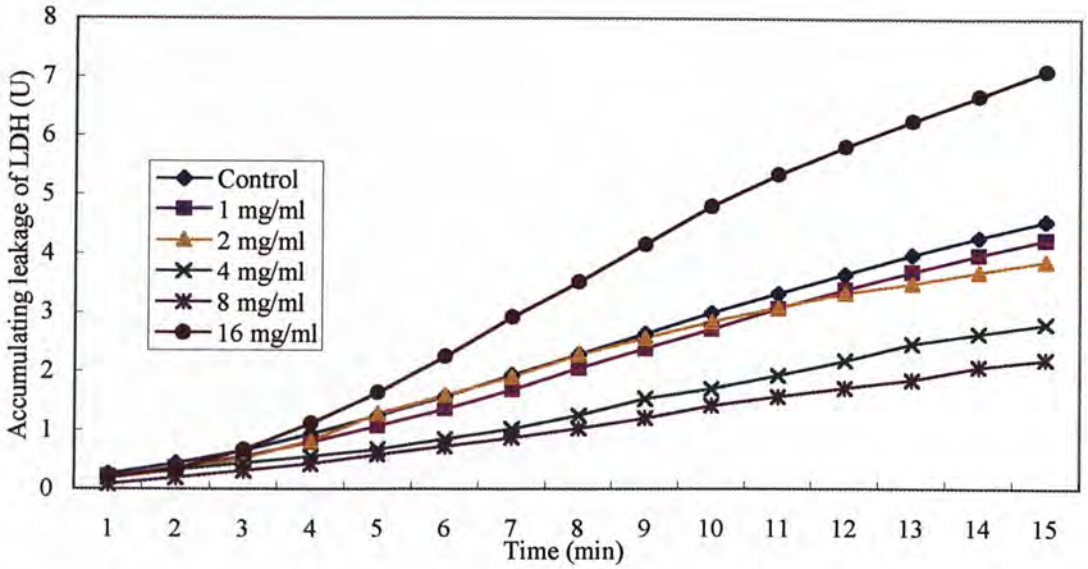


b)

Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	32% ± 8%	37% ± 17%	46% ± 6%	54% ± 18%	50% ± 27%	49% ± 17%
2	41% ± 12%	43% ± 23%	54% ± 8%	57% ± 14%	62% ± 23%	55% ± 23%
3	42% ± 15%	44% ± 22%	65% ± 21%	70% ± 25%	85% ± 25%	61% ± 22%
4	42% ± 18%	45% ± 23%	76% ± 35%	82% ± 27%	101% ± 46%	62% ± 20%
5	44% ± 18%	47% ± 28%	88% ± 43%	86% ± 26%	111% ± 39%	60% ± 18%
6	48% ± 19%	48% ± 28%	88% ± 49%	96% ± 31%	123% ± 27%	64% ± 20%
7	49% ± 16%	54% ± 24%	85% ± 48%	97% ± 29%	134% ± 15%	66% ± 24%
8	50% ± 16%	53% ± 24%	83% ± 42%	98% ± 32%	138% ± 17%	59% ± 24%
9	51% ± 16%	54% ± 25%	81% ± 38%	92% ± 27%	142% ± 4%	62% ± 23%
10	52% ± 16%	55% ± 24%	79% ± 35%	96% ± 22%	144% ± 5%	64% ± 23%
11	52% ± 16%	58% ± 26%	78% ± 35%	94% ± 22%	142% ± 4%	61% ± 24%
12	53% ± 17%	55% ± 26%	76% ± 31%	88% ± 29%	149% ± 2%	63% ± 21%
13	53% ± 17%	53% ± 31%	79% ± 35%	89% ± 23%	139% ± 6%	61% ± 26%
14	54% ± 17%	53% ± 29%	72% ± 24%	86% ± 22%	147% ± 14%	65% ± 26%
15	53% ± 17%	52% ± 30%	71% ± 23%	85% ± 20%	131% ± 16%	62% ± 24%

Figure 4.7. Coronary flow rate recovery of ischemia-reperfusion injured heart protected by Danshen at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)

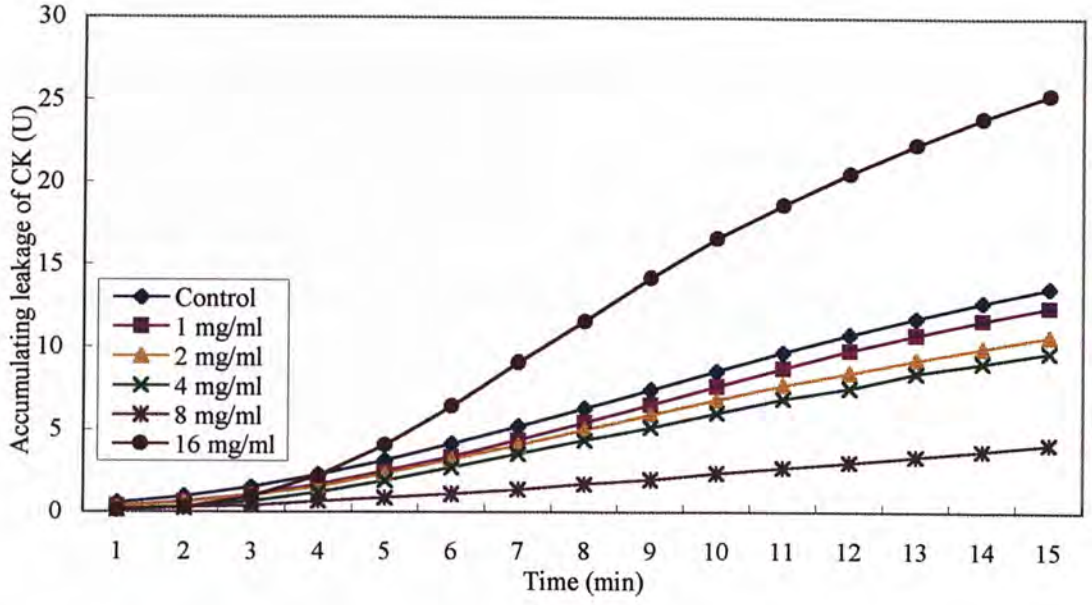


b)

	Accumulating leakage of LDH (U)					
Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	0.26 ± 0.12	0.21 ± 0.07	0.18 ± 0.07	0.20 ± 0.00	0.08 ± 0.01	0.20 ± 0.09
2	0.44 ± 0.13	0.35 ± 0.11	0.31 ± 0.10	0.33 ± 0.04	0.19 ± 0.04	0.34 ± 0.13
3	0.65 ± 0.14	0.54 ± 0.17	0.51 ± 0.17	0.44 ± 0.10	0.31 ± 0.09	0.67 ± 0.19
4	0.93 ± 0.16	0.79 ± 0.22	0.81 ± 0.19	0.55 ± 0.13	0.43 ± 0.11	1.12 ± 0.21
5	1.24 ± 0.18	1.06 ± 0.28	1.28 ± 0.20	0.67 ± 0.16	0.58 ± 0.12	1.64 ± 0.25
6	1.58 ± 0.21	1.36 ± 0.35	1.59 ± 0.01	0.85 ± 0.27	0.72 ± 0.08	2.25 ± 0.30
7	1.94 ± 0.23	1.68 ± 0.45	1.90 ± 0.08	1.02 ± 0.41	0.87 ± 0.08	2.92 ± 0.36
8	2.29 ± 0.28	2.05 ± 0.55	2.28 ± 0.14	1.26 ± 0.48	1.03 ± 0.03	3.51 ± 0.43
9	2.64 ± 0.35	2.39 ± 0.67	2.56 ± 0.17	1.53 ± 0.67	1.21 ± 0.04	4.15 ± 0.50
10	3.00 ± 0.43	2.72 ± 0.79	2.85 ± 0.20	1.71 ± 0.79	1.42 ± 0.07	4.80 ± 0.55
11	3.32 ± 0.53	3.07 ± 0.90	3.09 ± 0.27	1.93 ± 0.88	1.57 ± 0.16	5.34 ± 0.60
12	3.65 ± 0.63	3.39 ± 1.01	3.33 ± 0.28	2.18 ± 0.99	1.71 ± 0.17	5.81 ± 0.61
13	3.98 ± 0.69	3.68 ± 1.09	3.49 ± 0.32	2.47 ± 1.16	1.85 ± 0.26	6.26 ± 0.59
14	4.27 ± 0.77	3.97 ± 1.19	3.68 ± 0.34	2.64 ± 1.21	2.07 ± 0.39	6.68 ± 0.58
15	4.55 ± 0.80	4.23 ± 1.28	3.86 ± 0.37	2.80 ± 1.32	2.20 ± 0.45	7.11 ± 0.62

Figure 4.8. Accumulating leakage of lactate dehydrogenase of ischemia-reperfusion injured heart protected by Danshen at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)



b)

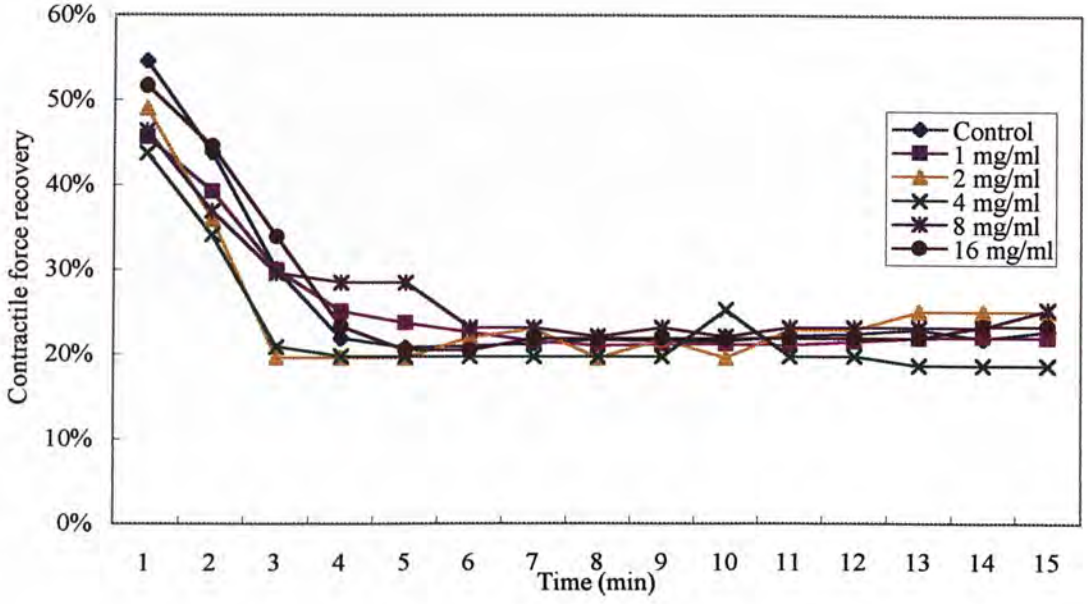
	Accumulating leakage of CK (U)					
Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	0.54± 0.29	0.31± 0.13	0.28± 0.12	0.13± 0.01	0.10± 0.05	0.08± 0.13
2	0.92± 0.34	0.60± 0.21	0.56± 0.19	0.31± 0.06	0.23± 0.10	0.25± 0.20
3	1.49± 0.37	1.02± 0.33	0.98± 0.33	0.64± 0.14	0.38± 0.15	0.90± 0.33
4	2.23± 0.43	1.65± 0.52	1.51± 0.53	1.18± 0.51	0.63± 0.18	2.18± 0.52
5	3.12± 0.56	2.45± 0.76	2.31± 0.85	1.90± 1.11	0.86± 0.23	4.06± 0.80
6	4.13± 0.74	3.33± 1.02	3.17± 1.21	2.69± 1.67	1.07± 0.23	6.44± 1.11
7	5.20± 0.97	4.36± 1.31	4.05± 1.69	3.51± 2.27	1.36± 0.28	9.08± 1.50
8	6.30± 1.23	5.44± 1.63	4.99± 2.19	4.35± 2.87	1.69± 0.27	11.55± 1.91
9	7.42± 1.54	6.52± 1.97	5.92± 2.70	5.15± 3.45	1.96± 0.28	14.20± 2.34
10	8.56± 1.91	7.62± 2.34	6.81± 3.25	6.01± 4.11	2.35± 0.21	16.61± 2.80
11	9.68± 2.37	8.69± 2.72	7.70± 3.89	6.88± 4.76	2.68± 0.27	18.63± 3.31
12	10.75± 2.74	9.77± 3.08	8.43± 4.30	7.53± 5.18	3.00± 0.24	20.54± 3.69
13	11.74± 3.08	10.71± 3.41	9.22± 4.74	8.41± 5.53	3.36± 0.14	22.30± 4.07
14	12.74± 3.42	11.62± 3.77	9.94± 5.21	9.05± 5.90	3.71± 0.01	23.93± 4.49
15	13.61± 3.70	12.41± 4.07	10.65± 5.70	9.67± 6.26	4.13± 0.13	25.35± 4.89

Figure 4.9. Accumulating leakage of creatine kinase of ischemia-reperfusion injured heart protected by Danshen at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

c) Gegen

As shown in figures 4.10, 4.11, 4.12 and 4.13, Gegen did not show obvious positive results to all four measured parameters, no enhancement on the recovery of both contractile force and coronary flow rate, and no reduction on the amount of leakage of both heart specific enzymes, i.e. lactate dehydrogenase and creatine kinase up to the dose of 16 mg/ml.

a)

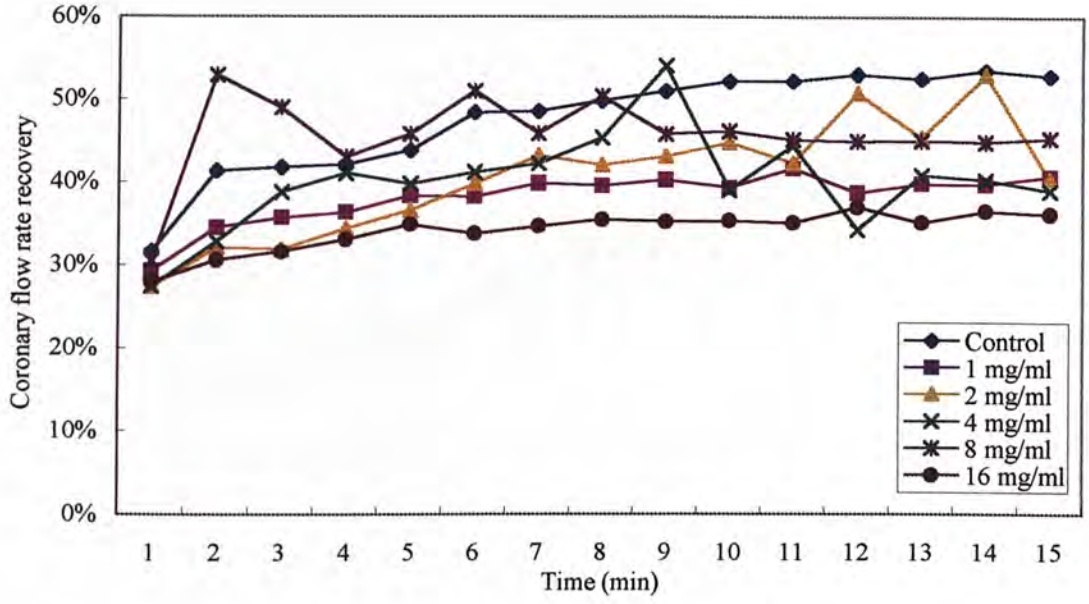


b)

Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	55% ± 18%	46% ± 27%	49% ± 19%	44% ± 22%	46% ± 15%	52% ± 20%
2	44% ± 17%	39% ± 22%	36% ± 16%	34% ± 16%	37% ± 18%	45% ± 13%
3	30% ± 11%	30% ± 9%	20% ± 10%	21% ± 9%	29% ± 10%	34% ± 13%
4	22% ± 8%	25% ± 6%	20% ± 11%	20% ± 9%	28% ± 8%	23% ± 8%
5	21% ± 7%	24% ± 6%	20% ± 8%	20% ± 9%	28% ± 8%	21% ± 10%
6	21% ± 6%	23% ± 6%	22% ± 7%	20% ± 9%	23% ± 9%	21% ± 10%
7	21% ± 6%	22% ± 9%	23% ± 7%	20% ± 8%	23% ± 9%	22% ± 7%
8	22% ± 5%	21% ± 8%	20% ± 7%	20% ± 8%	22% ± 7%	22% ± 8%
9	22% ± 6%	21% ± 8%	22% ± 7%	20% ± 8%	23% ± 7%	22% ± 9%
10	22% ± 6%	21% ± 8%	20% ± 8%	25% ± 13%	22% ± 10%	22% ± 9%
11	22% ± 6%	21% ± 7%	23% ± 8%	20% ± 9%	23% ± 11%	22% ± 8%
12	22% ± 6%	21% ± 7%	23% ± 8%	20% ± 8%	23% ± 12%	22% ± 8%
13	23% ± 6%	22% ± 7%	25% ± 9%	19% ± 8%	23% ± 13%	22% ± 8%
14	22% ± 9%	22% ± 7%	25% ± 9%	19% ± 8%	23% ± 10%	23% ± 9%
15	23% ± 10%	22% ± 7%	25% ± 11%	19% ± 13%	25% ± 6%	23% ± 9%

Figure 4.10. Contractile force recovery of ischemia-reperfusion injured heart protected by Gegen at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)

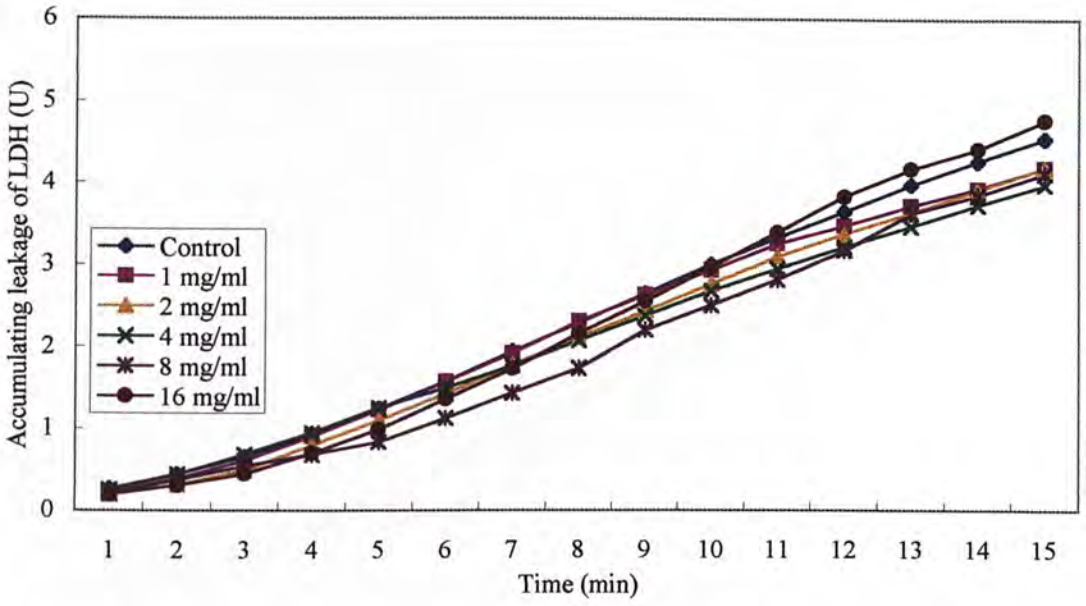


b)

Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	32% ± 8%	29% ± 10%	27% ± 9%	27% ± 19%	31% ± 24%	28% ± 22%
2	41% ± 12%	34% ± 11%	32% ± 12%	33% ± 13%	53% ± 22%	31% ± 18%
3	42% ± 15%	36% ± 15%	32% ± 15%	39% ± 14%	49% ± 20%	32% ± 17%
4	42% ± 18%	36% ± 21%	34% ± 20%	41% ± 14%	43% ± 18%	33% ± 16%
5	44% ± 18%	38% ± 21%	37% ± 20%	40% ± 18%	46% ± 24%	35% ± 21%
6	48% ± 19%	38% ± 21%	40% ± 20%	41% ± 21%	51% ± 29%	34% ± 25%
7	49% ± 16%	40% ± 21%	43% ± 19%	42% ± 20%	46% ± 27%	35% ± 24%
8	50% ± 16%	40% ± 22%	42% ± 19%	45% ± 21%	50% ± 29%	36% ± 25%
9	51% ± 16%	40% ± 21%	43% ± 19%	54% ± 21%	46% ± 25%	35% ± 23%
10	52% ± 16%	39% ± 21%	45% ± 18%	39% ± 19%	46% ± 23%	35% ± 21%
11	52% ± 16%	42% ± 19%	42% ± 18%	44% ± 20%	45% ± 22%	35% ± 21%
12	53% ± 17%	39% ± 21%	51% ± 19%	34% ± 19%	45% ± 19%	37% ± 19%
13	53% ± 17%	40% ± 21%	45% ± 19%	41% ± 17%	45% ± 17%	35% ± 17%
14	54% ± 17%	40% ± 20%	53% ± 19%	40% ± 17%	45% ± 17%	37% ± 17%
15	53% ± 17%	41% ± 21%	40% ± 19%	39% ± 14%	45% ± 13%	36% ± 14%

Figure 4.11. Coronary flow rate recovery of ischemia-reperfusion injured heart protected by Gegen at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)

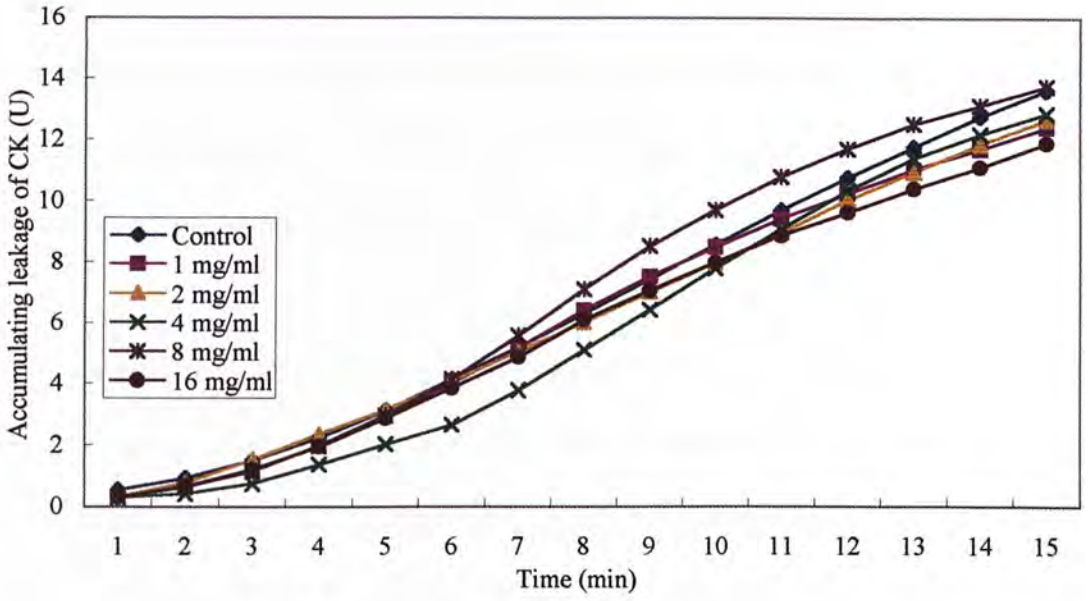


b)

	Accumulating leakage of LDH (U)					
Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	0.26 ± 0.12	0.21 ± 0.04	0.19 ± 0.04	0.25 ± 0.04	0.20 ± 0.04	0.19 ± 0.06
2	0.44 ± 0.13	0.38 ± 0.06	0.30 ± 0.07	0.43 ± 0.06	0.37 ± 0.06	0.30 ± 0.10
3	0.65 ± 0.14	0.60 ± 0.09	0.48 ± 0.10	0.68 ± 0.09	0.54 ± 0.09	0.44 ± 0.15
4	0.93 ± 0.16	0.90 ± 0.12	0.79 ± 0.11	0.95 ± 0.10	0.68 ± 0.10	0.70 ± 0.16
5	1.24 ± 0.18	1.23 ± 0.12	1.10 ± 0.13	1.25 ± 0.12	0.83 ± 0.12	0.99 ± 0.19
6	1.58 ± 0.21	1.57 ± 0.11	1.42 ± 0.15	1.49 ± 0.14	1.13 ± 0.14	1.36 ± 0.23
7	1.94 ± 0.23	1.91 ± 0.08	1.77 ± 0.18	1.76 ± 0.16	1.43 ± 0.17	1.73 ± 0.28
8	2.29 ± 0.28	2.30 ± 0.11	2.10 ± 0.22	2.07 ± 0.19	1.73 ± 0.20	2.16 ± 0.35
9	2.64 ± 0.35	2.62 ± 0.14	2.43 ± 0.25	2.37 ± 0.21	2.20 ± 0.23	2.55 ± 0.41
10	3.00 ± 0.43	2.92 ± 0.19	2.75 ± 0.28	2.68 ± 0.23	2.50 ± 0.25	2.97 ± 0.45
11	3.32 ± 0.53	3.24 ± 0.19	3.09 ± 0.30	2.95 ± 0.26	2.81 ± 0.28	3.39 ± 0.48
12	3.65 ± 0.63	3.47 ± 0.19	3.37 ± 0.30	3.21 ± 0.27	3.17 ± 0.29	3.83 ± 0.48
13	3.98 ± 0.69	3.72 ± 0.17	3.63 ± 0.30	3.46 ± 0.26	3.62 ± 0.28	4.17 ± 0.47
14	4.27 ± 0.77	3.93 ± 0.17	3.91 ± 0.29	3.73 ± 0.26	3.85 ± 0.27	4.42 ± 0.46
15	4.55 ± 0.80	4.18 ± 0.22	4.19 ± 0.31	3.98 ± 0.28	4.12 ± 0.29	4.77 ± 0.49

Figure 4.12. Accumulating leakage of lactate dehydrogenase of ischemia-reperfusion injured heart protected by Gegen at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)



b)

	Accumulating leakage of CK (U)					
Time	Control	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
1	0.54± 0.29	0.31± 0.21	0.30± 0.06	0.29± 0.11	0.29± 0.20	0.32± 0.04
2	0.92± 0.34	0.63± 0.27	0.76± 0.08	0.41± 0.18	0.64± 0.27	0.64± 0.06
3	1.49± 0.37	1.16± 0.35	1.52± 0.13	0.74± 0.31	1.16± 0.34	1.19± 0.11
4	2.23± 0.43	1.96± 0.48	2.34± 0.34	1.36± 0.51	1.96± 0.47	1.93± 0.33
5	3.12± 0.56	2.95± 0.66	3.13± 0.59	2.02± 0.79	2.95± 0.65	2.87± 0.57
6	4.13± 0.74	4.01± 0.88	4.08± 0.96	2.64± 1.10	4.14± 0.88	3.84± 0.94
7	5.20± 0.97	5.18± 1.14	5.04± 1.26	3.78± 1.48	5.58± 1.13	4.87± 1.25
8	6.30± 1.23	6.41± 1.43	6.02± 1.67	5.11± 1.89	7.11± 1.42	6.10± 1.66
9	7.42± 1.54	7.51± 1.76	6.99± 2.04	6.42± 2.32	8.52± 1.75	7.06± 2.02
10	8.56± 1.91	8.47± 2.13	7.97± 2.30	7.79± 2.78	9.69± 2.12	7.98± 2.28
11	9.68± 2.37	9.39± 2.54	8.93± 2.66	9.07± 3.29	10.77± 2.54	8.84± 2.64
12	10.75± 2.74	10.21± 2.91	10.01± 2.87	10.33± 3.68	11.67± 2.90	9.61± 2.86
13	11.74± 3.08	10.99± 3.24	10.91± 3.15	11.36± 4.06	12.49± 3.24	10.37± 3.13
14	12.74± 3.42	11.66± 3.60	11.82± 3.36	12.16± 4.48	13.09± 3.59	11.08± 3.35
15	13.61± 3.70	12.36± 3.89	12.62± 3.58	12.84± 4.87	13.72± 3.88	11.86± 3.57

Figure 4.13. Accumulating leakage of creatine kinase of ischemia-reperfusion injured heart protected by Gegen at various doses. a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

d) Comparison among the three samples

The dose response of three samples, 7:3 (D:G) compound formula, Danshen (D) and Gegen (G), was illustrated in the previous part of results. 7:3 (D:G) compound formula and Danshen showed high level of protection at the dose of 8 mg/ml, so the effectiveness of them was compared in this part at the dose 8 mg/ml.

As shown in figure 4.14, Gegen did not show any enhancement on the contractile force recovery, as it exhibited nearly the same percentage as the control at around 20%. While the 7:3 (D:G) compound formula and Danshen in the dose of 8 mg/ml showed nearly same contractile force recovery of about 60%. Almost the contractile force recovery of 7:3 (D:G) compound formula and Danshen were the same except in the first few minutes. Danshen gave a better percentage recovery at the third to fifth minutes.

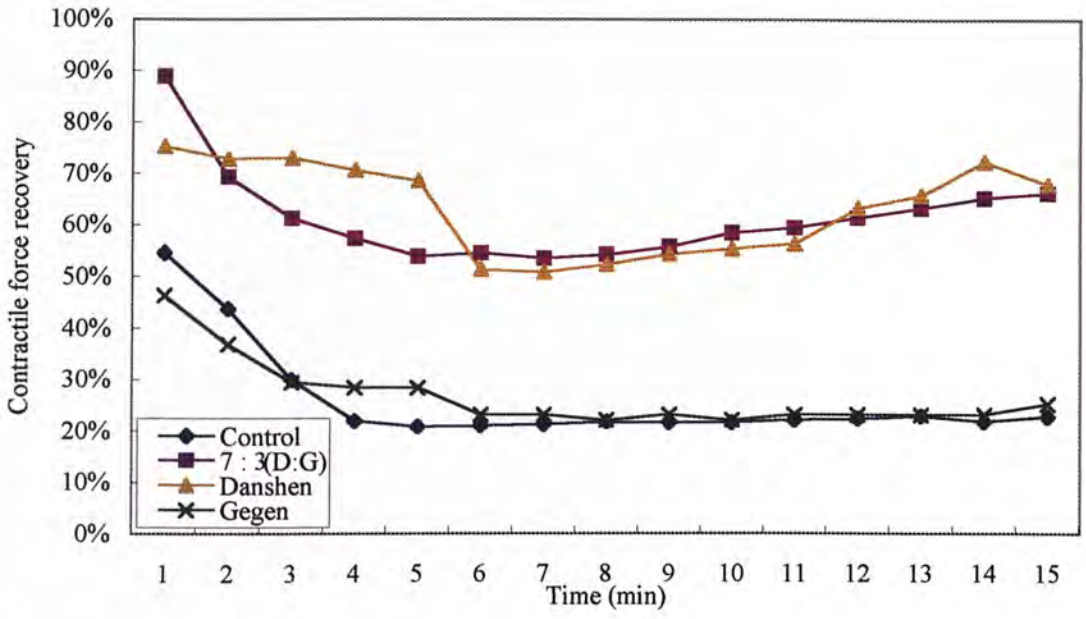
As shown in figure 4.15, Gegen did not show any enhancement on the coronary flow rate recovery, as it exhibited nearly the same percentage as the control at around 50%. While the 7:3 (D:G) compound formula and Danshen in the dose of 8 mg/ml showed nearly same contractile force recovery at the first four minutes of reperfusion that rose from 50% to 100%. After the fourth minute, the curve of 7:3 (D:G) compound formula became flattened at around 110% while the curve of Danshen continuously rose up to 140% at the ninth minute.

As shown in the figure 4.16, Gegen did show slightly reduction on the accumulating leakage of lactate dehydrogenase where 4.1 U of Gegen comparing to

4.5 U of control. The 7:3 (D:G) compound formula reduced the lactate dehydrogenase amount to 3.3 U. Danshen was the most effective one, it reduced the lactate dehydrogenase amount to 2.2 U. The protective effectiveness of them was clearly shown in descending order of Danshen, 7:3 (D:G) compound formula and Gegen in this part of lactate dehydrogenase amount measurement.

As shown in figure 4.17, Gegen did not show reduction on the accumulating leakage of creatine kinase where 13.7 U of Gegen comparing to 13.6 of control. The 7:3 (D:G) compound formula reduced the creatine kinase amount to 6.2 U. Danshen was the most effective one, it reduced the creatine kinase amount to 4.1 U. The protective effectiveness of them was clearly showed in descending order of Danshen, 7:3 (D:G) compound formula and Gegen in this part of creatine kinase amount measurement.

a)

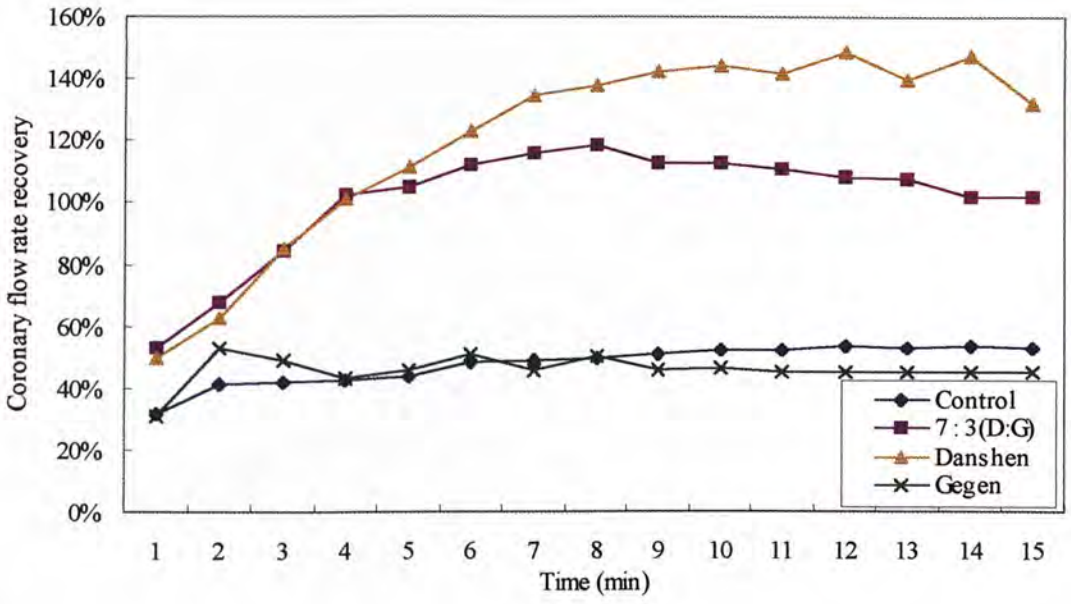


b)

	Contractile force recovery (%)			
Time	Control	7:3 (D:G)	Danshen	Gegen
1	0.55 ± 0.18	0.89 ± 0.07	0.75 ± 0.19	0.46 ± 0.15
2	0.44 ± 0.17	0.69 ± 0.27	0.73 ± 0.22	0.37 ± 0.18
3	0.30 ± 0.11	0.61 ± 0.24	0.73 ± 0.20	0.29 ± 0.10
4	0.22 ± 0.08	0.57 ± 0.21	0.71 ± 0.17	0.28 ± 0.08
5	0.21 ± 0.07	0.54 ± 0.19	0.68 ± 0.10	0.28 ± 0.08
6	0.21 ± 0.06	0.54 ± 0.20	0.51 ± 0.26	0.23 ± 0.09
7	0.21 ± 0.06	0.53 ± 0.16	0.51 ± 0.25	0.23 ± 0.09
8	0.22 ± 0.05	0.54 ± 0.16	0.52 ± 0.27	0.22 ± 0.07
9	0.22 ± 0.06	0.56 ± 0.16	0.54 ± 0.29	0.23 ± 0.07
10	0.22 ± 0.06	0.58 ± 0.16	0.55 ± 0.30	0.22 ± 0.10
11	0.22 ± 0.06	0.59 ± 0.17	0.56 ± 0.31	0.23 ± 0.11
12	0.22 ± 0.06	0.61 ± 0.18	0.63 ± 0.24	0.23 ± 0.12
13	0.23 ± 0.06	0.63 ± 0.19	0.66 ± 0.27	0.23 ± 0.13
14	0.22 ± 0.09	0.65 ± 0.18	0.72 ± 0.21	0.23 ± 0.10
15	0.23 ± 0.10	0.66 ± 0.18	0.68 ± 0.29	0.25 ± 0.06

Figure 4.14. Comparison of the protective effect on the contractile force recovery of 7:3 (D:G) compound formula, Danshen (D) and Gegen (G). a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)

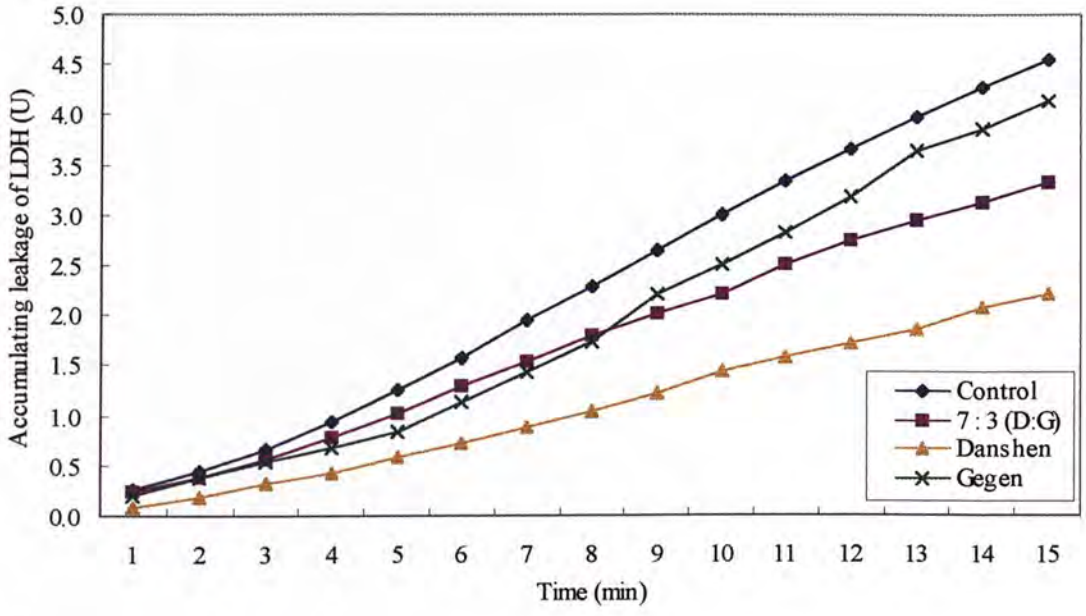


b)

	Coronary flow rate recovery (%)			
Time	Control	7:3 (D:G)	Danshen	Gegen
1	0.32 ± 0.08	0.53 ± 0.27	0.50 ± 0.27	0.31 ± 0.24
2	0.41 ± 0.12	0.67 ± 0.25	0.62 ± 0.23	0.53 ± 0.22
3	0.42 ± 0.15	0.84 ± 0.23	0.85 ± 0.25	0.49 ± 0.20
4	0.42 ± 0.18	1.02 ± 0.21	1.01 ± 0.46	0.43 ± 0.18
5	0.44 ± 0.18	1.05 ± 0.27	1.11 ± 0.39	0.46 ± 0.24
6	0.48 ± 0.19	1.12 ± 0.32	1.23 ± 0.27	0.51 ± 0.29
7	0.49 ± 0.16	1.16 ± 0.30	1.34 ± 0.15	0.46 ± 0.27
8	0.50 ± 0.16	1.18 ± 0.32	1.38 ± 0.17	0.50 ± 0.29
9	0.51 ± 0.16	1.12 ± 0.28	1.42 ± 0.04	0.46 ± 0.25
10	0.52 ± 0.16	1.12 ± 0.26	1.44 ± 0.05	0.46 ± 0.23
11	0.52 ± 0.16	1.11 ± 0.25	1.42 ± 0.04	0.45 ± 0.22
12	0.53 ± 0.17	1.08 ± 0.22	1.49 ± 0.02	0.45 ± 0.19
13	0.53 ± 0.17	1.07 ± 0.20	1.39 ± 0.06	0.45 ± 0.17
14	0.54 ± 0.17	1.01 ± 0.20	1.47 ± 0.14	0.45 ± 0.17
15	0.53 ± 0.17	1.02 ± 0.16	1.31 ± 0.16	0.45 ± 0.13

Figure 4.15. Comparison of the protective effect on the coronary flow rate recovery of 7:3 (D:G) compound formula, Danshen (D) and Gegen (G). a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)

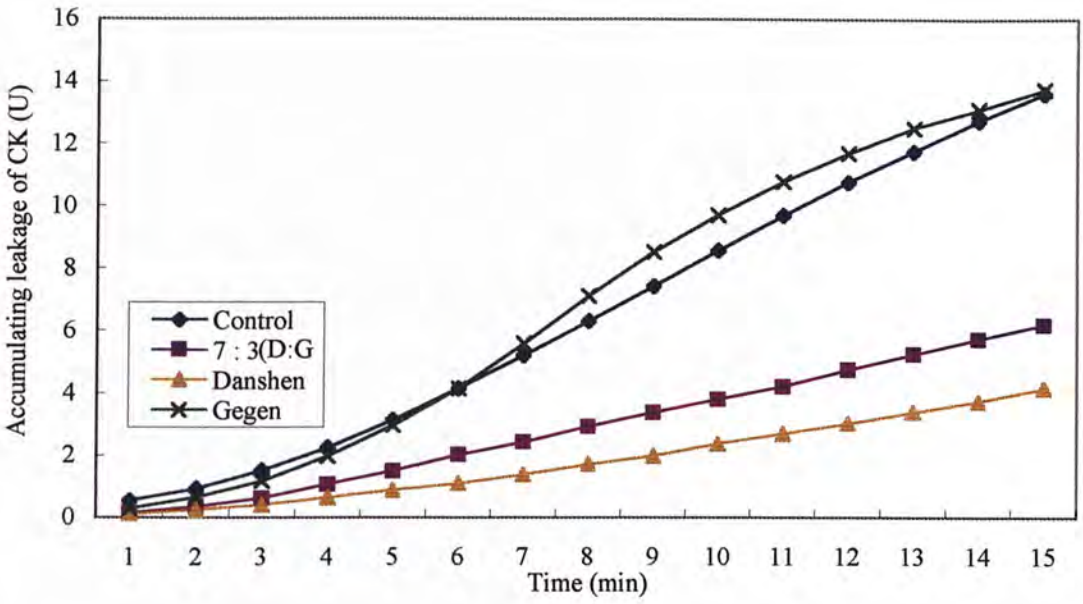


b)

	Accumulating leakage of LDH (U)			
Time	Control	7:3 (D:G)	Danshen	Gegen
1	0.26 ± 0.12	0.23 ± 0.09	0.08 ± 0.01	0.20 ± 0.04
2	0.44 ± 0.13	0.37 ± 0.11	0.19 ± 0.04	0.37 ± 0.06
3	0.65 ± 0.14	0.55 ± 0.15	0.31 ± 0.09	0.54 ± 0.09
4	0.93 ± 0.16	0.78 ± 0.18	0.43 ± 0.11	0.68 ± 0.10
5	1.24 ± 0.18	1.01 ± 0.20	0.58 ± 0.12	0.83 ± 0.12
6	1.58 ± 0.21	1.29 ± 0.26	0.72 ± 0.08	1.13 ± 0.14
7	1.94 ± 0.23	1.53 ± 0.25	0.87 ± 0.08	1.43 ± 0.17
8	2.29 ± 0.28	1.78 ± 0.25	1.03 ± 0.03	1.73 ± 0.20
9	2.64 ± 0.35	1.99 ± 0.25	1.21 ± 0.04	2.20 ± 0.23
10	3.00 ± 0.43	2.21 ± 0.27	1.42 ± 0.07	2.50 ± 0.25
11	3.32 ± 0.53	2.49 ± 0.34	1.57 ± 0.16	2.81 ± 0.28
12	3.65 ± 0.63	2.74 ± 0.40	1.71 ± 0.17	3.17 ± 0.29
13	3.98 ± 0.69	2.94 ± 0.37	1.85 ± 0.26	3.62 ± 0.28
14	4.27 ± 0.77	3.11 ± 0.38	2.07 ± 0.39	3.85 ± 0.27
15	4.55 ± 0.80	3.32 ± 0.43	2.20 ± 0.45	4.12 ± 0.29

Figure 4.16. Comparison of the protective effect on the accumulating leakage of lactate dehydrogenase of 7:3 (D:G) compound formula, Danshen (D) and Gegen (G). a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

a)



b)

	Accumulating leakage of CK (U)			
Time	Control	7:3 (D:G)	Danshen	Gegen
1	0.54 ± 0.29	0.13 ± 0.05	0.10 ± 0.05	0.29 ± 0.20
2	0.92 ± 0.34	0.34 ± 0.09	0.23 ± 0.10	0.64 ± 0.27
3	1.49 ± 0.37	0.61 ± 0.16	0.38 ± 0.15	1.16 ± 0.34
4	2.23 ± 0.43	1.05 ± 0.26	0.63 ± 0.18	1.96 ± 0.47
5	3.12 ± 0.56	1.47 ± 0.37	0.86 ± 0.23	2.95 ± 0.65
6	4.13 ± 0.74	2.00 ± 0.43	1.07 ± 0.23	4.14 ± 0.88
7	5.20 ± 0.97	2.41 ± 0.60	1.36 ± 0.28	5.58 ± 1.13
8	6.30 ± 1.23	2.91 ± 0.73	1.69 ± 0.27	7.11 ± 1.42
9	7.42 ± 1.54	3.35 ± 0.89	1.96 ± 0.28	8.52 ± 1.75
10	8.56 ± 1.91	3.78 ± 1.02	2.35 ± 0.21	9.69 ± 2.12
11	9.68 ± 2.37	4.19 ± 1.15	2.68 ± 0.27	10.77 ± 2.54
12	10.75 ± 2.74	4.72 ± 1.21	3.00 ± 0.24	11.67 ± 2.90
13	11.74 ± 3.08	5.22 ± 1.36	3.36 ± 0.14	12.49 ± 3.24
14	12.74 ± 3.42	5.70 ± 1.43	3.71 ± 0.01	13.09 ± 3.59
15	13.61 ± 3.70	6.17 ± 1.57	4.13 ± 0.13	13.72 ± 3.88

Figure 4.17. Comparison of the protective effect on the accumulating leakage of creatine kinase of 7:3 (D:G) compound formula, Danshen (D) and Gegen (G). a) Curves were plotted by the mean of 3 rat hearts per group. b) Data are tabulated by mean ± SD of 3 rat hearts for each group.

4.2.3 Discussion

The 7:3 (D:G) compound formula was not as protective as Danshen in the ischemia-reperfusion rat heart model that demonstrated in the heart specific enzyme studies. Although Gegen did not show positive protective effects, at least the protective effect of 7:3 (D:G) compound formula did not show a sudden drop due to the presence of Gegen. Gegen did not show positive protection in this part. However, the 7:3 (D:G) compound formula showed similar protective effect to Danshen giving around 60% contractile force recovery. As 7:3 (D:G) compound formula was extracted by a mixture which only contained 70% Danshen by mass, there may be some supplementary effects coming from the Gegen ingredient or some compounds in Gegen can enhance the action of Danshen to restore the contractile force.

Patients having myocardial infraction may suffer from ischemia-reperfusion heart injury. The protective effect of the 7:3 (D:G) compound formula may be useful as it showed a very good contractile force recovery and coronary flow recovery. It also reduced the amount of heart cells damage which was reflected by the lactate dehydrogenase and creatine kinase studies.

Patients after angioplasty procedure may also form a case of reperfusion injury to the heart. The 7:3 (D:G) compound formula may also be useful for them. Further clinical studies are needed to prove this hypothesis.

Chapter 5 Vasodilation study

Only 6 years after the discovery of endothelial-derived relaxing factor (EDRF), it became apparent that many cardiovascular diseases are associated with an impairment of endothelium-dependent vasorelaxation. This has been shown in hypercholesterolemic rabbits and monkeys and in patients having coronary artery disease or typical risk factors predisposing to this condition. Endothelium-dependent vasorelaxation is also abnormal in other disease states such as heart failure, diabetes mellitus and hypertension (Moncada and Higgs, 1993). Presumably, there is a loss of endothelial production and/or bioavailability of NO (nitric oxide, nitrogen monoxide) in these disorders.

Vasodilation is the best documented activity of NO in the cardiovascular system. This action led to the discovery of endothelium-derived relaxing factor 20 years ago (Furchgott and Zawadzki, 1980). Subsequent research has shown that endogenous NO production is involved in the regulation of local vasomotion and blood pressure. Numerous conditions characterized by an impaired availability of NO have been found to be associated with enhanced synthesis of endothelin-1 (ET-1) as a potent endogenous vasoconstrictor (Rossi *et al.*, 2001). Pharmacological inhibition of endogenous NO synthesis has been shown to induce a rise in blood pressure in man (Stamler *et al.*, 1994) and disruption of the endogenous NO synthase (eNOS) gene by the `knock-out' technique causes mild hypertension in mice (Huang *et al.*, 1995). Elevated blood pressure is a well known risk factor for the development of cardiovascular diseases such as stroke and myocardial infarction, while a reduction of blood pressure is effective in reducing morbidity and mortality of cardiovascular

diseases (Kannel, 2000). Thus, maintenance of normal blood pressure by endothelial NO may be considered as part of its vasoprotective action. Therefore the vasodilation effects of drugs are of interest and need to be investigated.

NO is a soluble gas that diffuses freely in both water and lipid and has a half-life of only a few seconds. It is synthesized from the amino acid L-arginine by nitric oxide synthase (NOS) (Palmer *et al.*, 1987). Two main forms of vascular NOS have been described, namely the calcium calmodulin-dependent constitutive NOS (eNOS) present in endothelial cells and the calcium-independent inducible NOS (iNOS). The iNOS is primarily expressed in immune cells and vascular smooth muscle cells (VSMC). NO diffuses to the VSMC inducing the formation of cyclic guanosine monophosphate. This, in turn, activates protein kinase C resulting in a decline in intracellular ionized calcium in the VSMC leading to relaxation and vasodilation (Moncada *et al.*, 1993 and Moncada *et al.*, 1991). The basal NO production can be upregulated by physical forces, such as shear stress, as well as by several receptor-operating transmitters and hormones such as estrogen, acetylcholine, bradykinin, substance P, serotonin, adrenaline and noradrenaline, adenosine and thromboxane (Lüscher and Noll 1995 ; Rubanyi *et al.*, 1986).

Therefore the involvment of endothelium and nitric oxide of the vasodilation effect of the 7:3 (D:G) compound formula were also interested for investigation in the second part of this chapter.

5.1 Vasodilation in organ bath

For long-term prevention of cardiovascular disease, controlling the blood pressure is very important. Higher blood pressure represents higher risk of atherosclerosis as higher turbulence occurs in the blood vessel (Spence *et al.*, 1984). In addition, cardiovascular diseases are always complicated with impaired blood vessel endothelium function and loss of vasodilation ability at certain degree. Restoration of vasodilation ability is beneficial to hypertension patients (Antony *et al.*, 1996).

The vasodilation effect of 7:3 (D:G) compound formula, Danshen aqueous and Gegen aqueous extract were investigated by the *ex-vivo* organ bath setup.

5.1.1 Materials and Methods

Chemicals

(R)-(-)-phenylephrine hydrochloride (Phe), acetylcholine chloride (Ach) and 9,11-dideoxy-9α,11α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619) were purchased from Sigma. Krebs solution (118.0 mM NaCl, 4.7 mM KCl, 1.64 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.55 mM D-glucose, 25.0 mM $NaHCO_3$ and 2.5 mM $CaCl_2$) was adjusted to pH 7.4 and filtered.

Amimal

Aorta was excised from 300 g male Sprague-Dawley (SD) rats and placed in Krebs solution. After fat and connective tissue were removed, aorta was cut into 1

mm width rings. The isolated aorta rings were mounted between two wires in organ bath. The condition was maintained at 37℃ Krebs solution and was gassed with mixture of 95% O_2 and 5% CO_2. The tension was slowly adjusted mechanically up to 1 g with an increasing rate not more then 0.2 g each 5 minute intervals. Finally 1 g basal tension was applied to the aorta rings.

Pre-test

The validity of the aorta rings was confirmed by contraction with 0.3 μM phenylephrine and then relaxation with 0.3 μM acetylcholine. If 70% or more relaxation was shown when comparing to the 0.3 μM phenylephrine induced contraction, the aorta ring was defined to be suitable for further use as the endothelial layer remained intact. Aorta ring was then equilibrated for an hour with three times changing Krebs solution before the test of samples.

Drug test

The aorta rings were contracted by 10 nM U46619, which is a potent prostaglandin H_2 receptor agonist, for 20 minutes. Samples were then added to the organ bath and the vasomotion were monitored throughout the experiment. Samples were prepared by Krebs solution.

Measurement

Tensions were recorded by MacLab data acquisition system through Grass force-displacement transducers, FT-03E. Data was recorded by a sampling rate of 10 per second. The background noise of raw data was then removed by triangular (Bartlett) window type of smoothing with window size of 201 points width before

analysis. Finally, results were expressed as percentage relaxation response of the maximal contraction induced by 10 nM U46619.

5.1.2 Results

Dose response vasodilation of aqueous extract of 7:3 (D:G) compound formula, Danshen and Gegen were investigated.

a) 7:3 (D:G) compound formula

Effects of formula concentrations on vasodilation were investigated at 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml. The vasodilation effect of them was very slow at the low doses, namely 0.5 mg/ml and 1 mg/ml, while faster responses were given at higher doses. Dose dependent manner could be clearly observed from the screenshot of the computerized MacLab system as shown in figure 5.1. The time taken to obtain 70% relaxation from maximum U44619 induced vasoconstriction was compared as illustrated in table 5.1. The time for 70% relaxation was reduced from 108 minutes to 14 minutes when the dose increased from 0.5 mg/ml to 4 mg/ml. Rate of vasodilation was also shown in table 5.1 and it was calculated by 70% divided by the time taken to give 70% relaxation. The unit of the rate was defined as percentage relaxed per minute. A plot of rate relaxation against 7:3 (D:G) formula concentration was shown in figure 5.2. Linear regression was indicated as R^2=0.992, the linear relationship was very clearly shown.

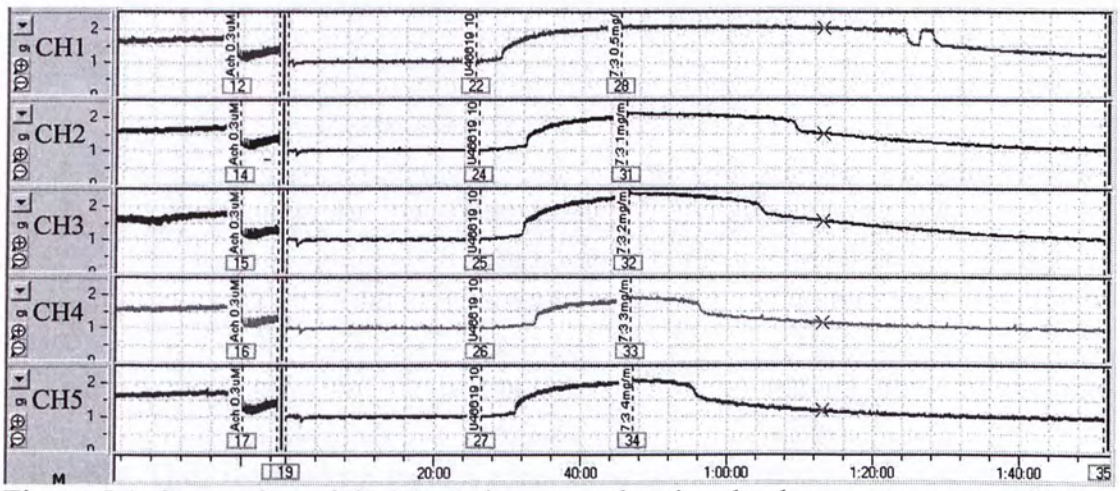


Figure 5.1. Screenshot of the MacLab system showing the dose response vasodilation of the 7:3 (D:G) compound formulae. Vertical dimension represents the tension force of the aorta ring in the unit of gram (g) and the horizontal dimension represents the time course during the experiment. Formula concentrations 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml were respectively added in channel 1 to 5. Dose dependent manner can be clearly observed.

Conc. (mg/ml)	Time (min)	Rate (%min^{-1})
0.5	108±12.29	0.66±0.08
1	58±3.56	1.21±0.07
2	32±2.50	2.18±0.17
3	18±1.71	3.86±0.38
4	14±1.41	5.04±0.55

Table 5.1. Time required for various concentration of 7:3 (D:G) compound formula to give a 70% vasodilation. Rate represents 70%/time, that is the percentage relax per minute. Data are mean ± SD of 4 aorta rings each group.

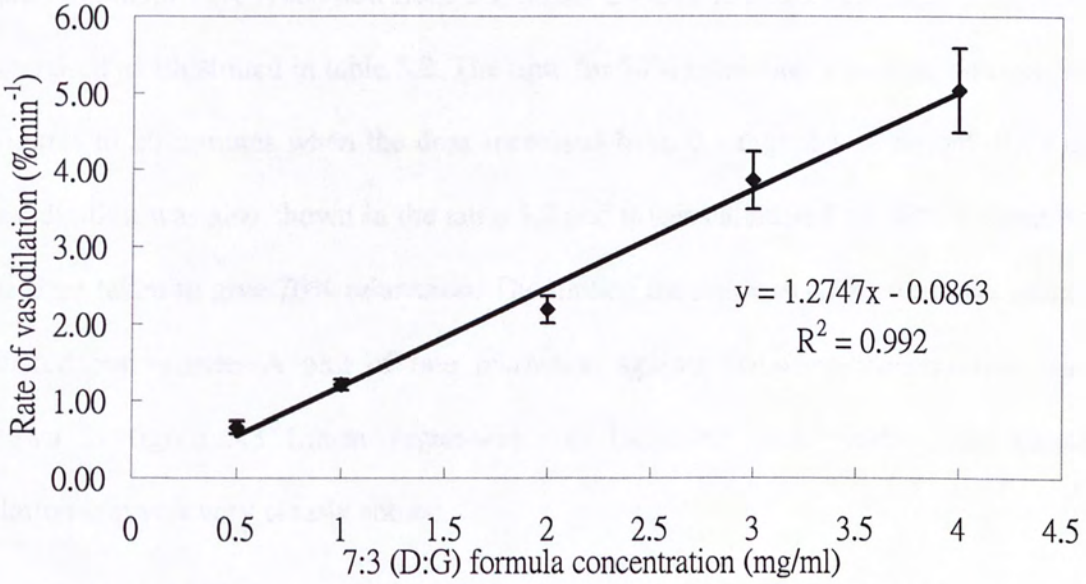


Figure 5.2. Linear dose-relaxation relationship between vasodilation rate and 7:3 (D:G) formula concentration. Data are mean ± SD of 4 aorta rings each group. R^2=0.992 implies linear relationship obtained.

b) Danshen aqueous extract

Effects of Danshen concentrations on vasodilation were investigated at 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml. The vasodilation effect of them was very slow at the low doses, namely 0.5 mg/ml and 1 mg/ml, while faster responses were given at higher doses. Dose dependent manner could be clearly observed from the screenshot of the computerized MacLab system as shown in figure 5.3. The time taken to obtain 70% relaxation from maximum U44619 induced vasoconstriction was compared as illustrated in table 5.2. The time for 70% relaxation was reduced from 99 minutes to 20 minutes when the dose increased from 0.5 mg/ml to 4 mg/ml. Rate of vasodilation was also shown in the table 5.2 and it was calculated by 70% divided by the time taken to give 70% relaxation. The unit of the rate was defined as percentage relaxed per minute. A plot of rate relaxation against Danshen concentration was shown in figure 5.3 Linear regression was indicated as R^2=0.9944, the linear relationship was very clearly shown.

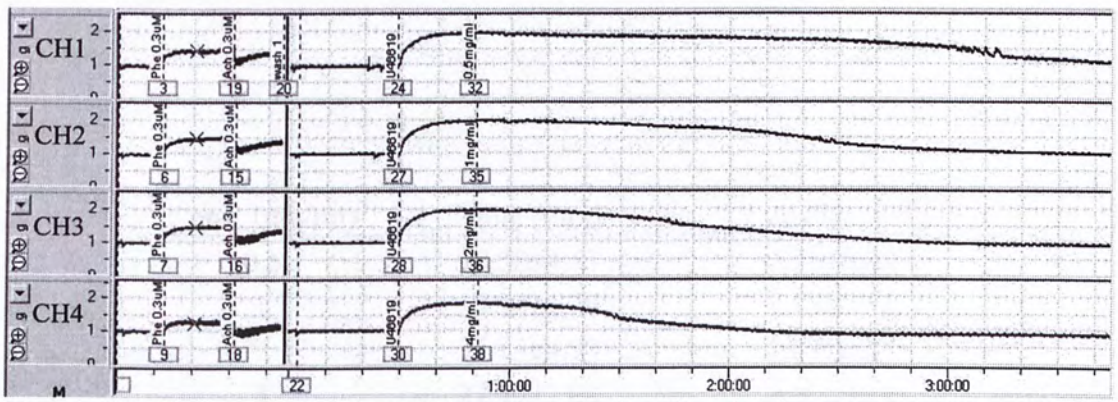


Figure 5.3. Screenshot of the MacLab system showing the dose response vasodilation of the Danshen aqueous extract. Vertical dimension represents the tension force of the aorta ring in the unit of gram (g) and the horizontal dimension represents the time course during the experiment. Formula concentrations 0.5 mg/ml, 1 mg/ml, 2 mg/ml and 4 mg/ml were respectively added in channel 1 to 4. Dose dependent manner can be clearly observed.

Conc. (mg/ml)	Time (min)	Rate (%min-1)
0.5	98.50±4.51	0.71±0.03
1	60.25±6.40	1.17±0.13
2	37.25±4.93	1.89±0.25
3	27.50±2.08	2.56±0.19
4	19.75±1.71	3.56±0.30

Table 5.2. Time required for various concentration of Danshen aqueous extract to give a 70% vasodilation. Rate represents 70%/time, that is the percentage relax per minute. Data are mean ± SD and of 4 aorta rings each group.

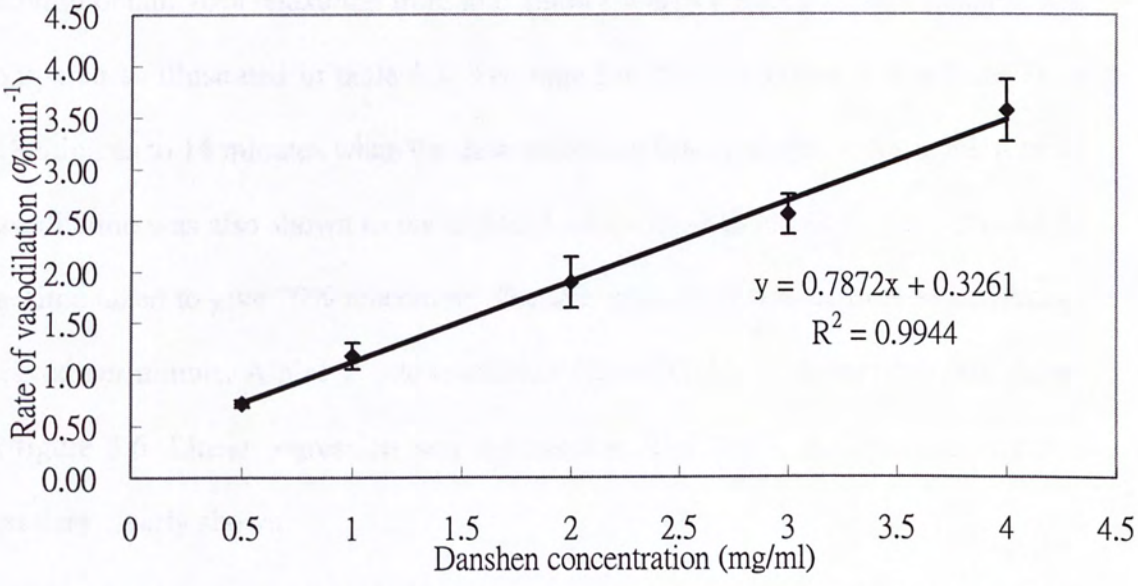


Figure 5.4. Linear dose-relaxation relationship between vasodilation rate and Danshen aqueous extract concentration. Data are mean ± SD and of 4 aorta rings each group. R^2=0.9944 implies linear relationship obtained.

c) Gegen aqueous extract

Effects of Gegen concentrations on vasodilation were investigated at 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 8 mg/ml. The vasodilation effect of them was very slow at the low doses, namely 1 mg/ml and 2 mg/ml while faster responses were given at higher doses. Dose dependent manner could be clearly observed from the screenshot of the computerized MacLab system as shown in figure 5.5. The time taken to obtain 70% relaxation from maximum U44619 induced vasoconstriction was compared as illustrated in table 5.3. The time for 70% relaxation was reduced from 118 minutes to 14 minutes when the dose increased from 1 mg/ml to 8 mg/ml. Rate of vasodilation was also shown in the table 5.3 and it was calculated by 70% divided by the time taken to give 70% relaxation. The unit of the rate was defined as percentage relaxed per minute. A plot of rate relaxation against Gegen concentration was shown in figure 5.6. Linear regression was indicated as R^2=0.9903, the linear relationship was very clearly shown.

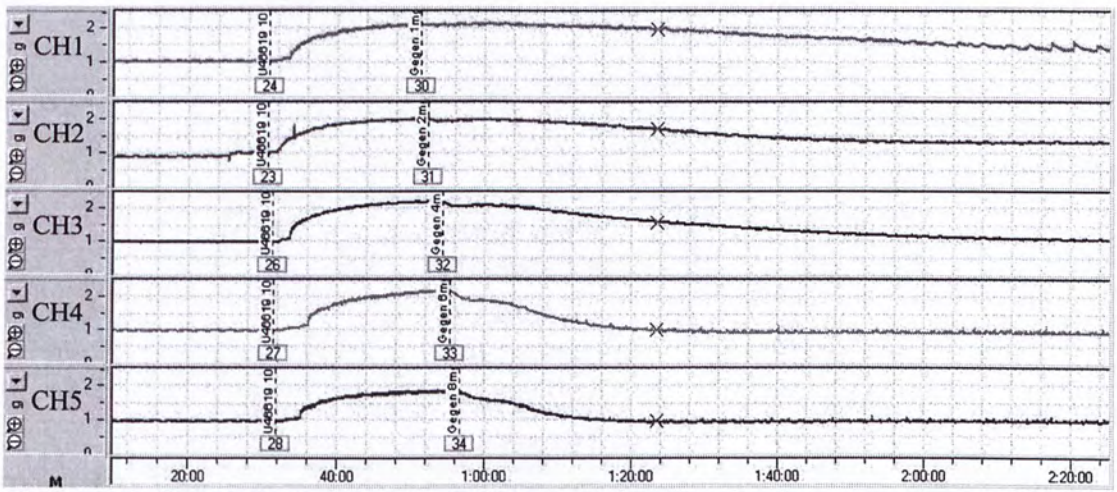


Figure 5.5. Screenshot of the MacLab system showing the dose response vasodilation of the Gegen extract. Vertical dimension represents the tension force of the aorta ring in the unit of gram (g) and the horizontal dimension represents the time course during the experiment. Formula concentrations 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml and 8 mg/ml were respectively added in channel 1 to 5. Dose dependent manner can be clearly observed.

Conc.	Time (min)	Rate (%min^{-1})
1	117.50±13.12	0.60±0.07
2	63.00±8.29	1.13±0.16
4	29.75±3.30	2.37±0.26
6	16.75±1.71	4.21±0.42
8	13.75±1.71	5.15±0.62

Table 5.3. Time required for various concentration of Gegen aqueous extract to give 70% vasodilation. Rate represents 70%/time, that is the percentage relax per minute. Data are mean ± SD of 4 aorta rings each group.

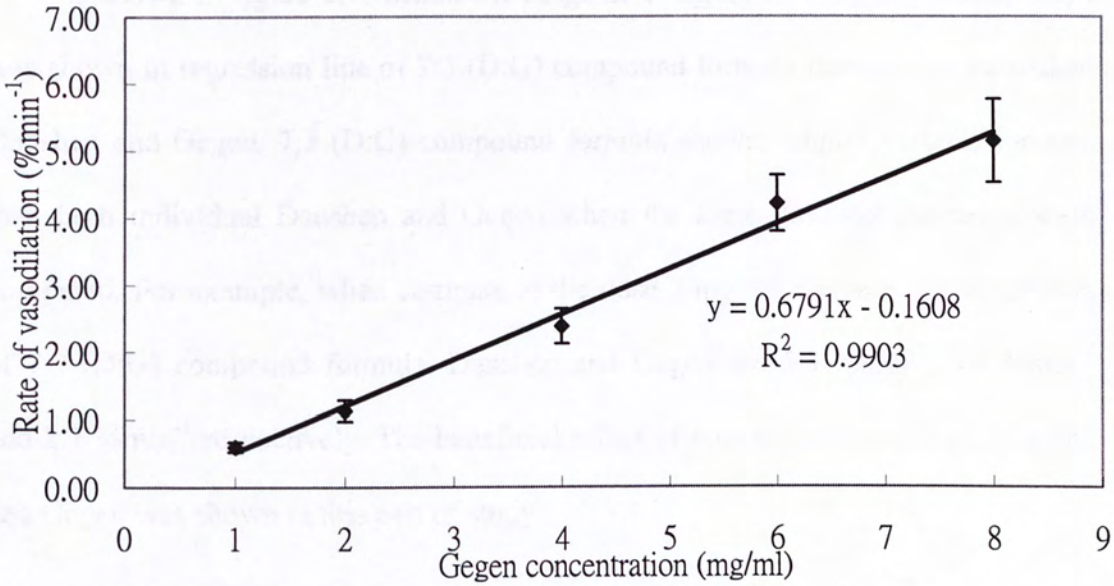


Figure 5.6. Linear dose-relaxation relationship between vasodilation rate and Gegen aqueous extract concentration. Data are mean ± SD of 4 aorta rings each group. R^2=0.9903 implies linear relationship obtained.

d) Comparison among the three samples

The dose response of the three samples, 7:3 (D:G) compound formula, Danshen (D) and Gegen (G), was illustrated in the previous part of results individually. The effectiveness of them can be compared by combining the graphs in figures 5.2, 5.4 and 5.6.

As shown in figure 5.7, within the range of 1 mg/ml to 4 mg/ml, greater slope was shown in regression line of 7:3 (D:G) compound formula than that of individual Danshen and Gegen. 7:3 (D:G) compound formula showed higher vasodilation rate than both individual Danshen and Gegen when the same dose concentration were compared. For example, when compare at the dose 3 mg/ml, the rate of vasodilation of 7:3 (D:G) compound formula, Danshen and Gegen are 5.3 $\%min^{-1}$, 3.8 $\%min^{-1}$ and 2.7 $\%min^{-1}$ respectively. The beneficial effect of compound formula of Danshen and Gegen was shown in this part of study.

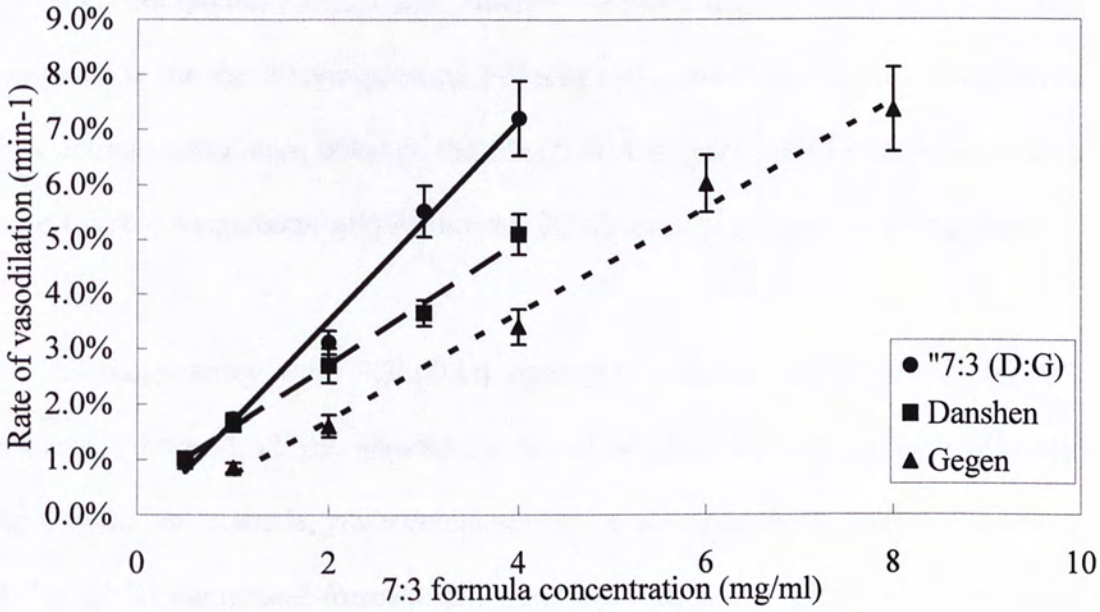


Figure 5.7. Comparison of the vasodilation effect among 7:3 (D:G) compound formula, Danshen (D) and Gegen (G). For the dose 3 mg/ml, the rate of vasodilation of 7:3 (D:G) compound formula, Danshen and Gegen are 5.3 $\%min^{-1}$, 3.8 $\%min^{-1}$ and 2.7 $\%min^{-1}$ respectively where the potency were 7:3 (D:G) compound formula, Danshen and Gegen in descending order. Data are mean ± SD of 4 aorta rings each group.

5.1.3 Discussion

All the three samples 7:3 (D:G) compound formula, Danshen (D) and Gegen (G) gave vasodilation effect to the isolated SD rat aorta rings. The mode of effects of them was slow acting and it is very different from other known vasodilating drugs. Therefore, the accumulating dose response cannot be studied and the time taken of various concentrations to obtain 70% relaxation of single dose was studied instead. 7:3 (D:G) compound formula and Danshen showed a faster effect than Gegen when compared at the same concentration. For long-term cardiovascular tonic supplement, slow acting vasodilation effect of the 7:3 (D:G) compound formula is believed to be more suitable for patients with cardiovascular disease rather than fast acting drugs.

As the potency were 7:3 (D:G) compound formula, Danshen and Gegen in descending order, it clearly showed the beneficial effect of combing use of Danshen and Gegen. For example, when compare at the dose 3 mg/ml, the rate of vasodilation of 7:3 (D:G) compound formula, Danshen and Gegen are 5.3 $\%min^{-1}$, 3.8 $\%min^{-1}$ and 2.7 $\%min^{-1}$ respectively. In addition, the combine effect was not only in an addition of effect from Danshen and Gegen, but in fact the beneficial effect was in a multiplied way. It was because 7:3 (D:G) compound formula only extracted from 70% Danshen plus 30% Gegen, but its effect is much higher than the sum effect of 70% individual Danshen and 30% Gegen when compared in terms of rate of vasodilation in the unit of $\%min^{-1}$. The synergistic effect of 7:3 (D:G) compound was observed in this part of experiment.

5.2 Endothelium dependent vasodilation

Pharmaceutical agents have different effects on endothelial function. Recently, von zur Mühlen and co-workers demonstrated that angiotensin converting enzyme (ACE) inhibition improved endothelium dependent vasodilation both in patients with untreated hypertension and in normotensive patients (von zur Mühlen *et al.*, 2001). They also found that an angiotensin-II subtype-receptor antagonist, irbesartan, and β_1-selective adrenoreceptor antagonist, atenolol, improve endothelium dependent vasodilation (von zur Mühlen *et al.*, 2001). Statins and other lipid-lowering drugs, such as cholestyramine, have been shown to have beneficial effects on endothelium dependent vasodilation in hypercholesterolemic patients (Duffy *et al.* 2001).

In order to investigate whether the effect of Danshen and Gegen on the vasodilation is an endothelium dependent vasodilation, the effect of them were tested on aorta without endothelium and aorta pre-treated in nitric oxide synthase inhibitor separately.

5.2.1 Materials and Methods

Chemicals

(R)-(-)-phenylephrine hydrochloride (Phe), acetylcholine chloride (Ach), 9,11-dideoxy-9α,11α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619) and N^G-nitro-L-arginine (L-NNA) were purchased from Sigma. Krebs solution (118.0 mM NaCl, 4.7 mM KCl, 1.64 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.55 mM D-glucose, 25.0 mM $NaHCO_3$ and 2.5 mM $CaCl_2$) was adjusted to pH 7.4 and filtered.

Animal

Aorta was excised from 300 g male Sprague-Dawley (SD) rats and placed in Krebs solution. After fat and connective tissue were removed, aorta was cut into 1 mm width rings. The isolated aorta rings were suspended between two stainless steel hooks in organ bath. The condition was maintained at 37℃ Krebs solution and was gassed with mixture of 95% O_2 and 5% CO_2. The tension was slowly adjusted mechanically up to 1 g with an increasing rate not more then 0.2 g each 5 minute intervals. Finally 1 g basal tension was applied to the aorta rings.

Removal of endothelial layer

The endothelium was removed by using forceps gently rubbing the intimal surface of the aorta on the hand with a clean glove. The glove was pre-moistened with Krebs solution before handling the aorta rings.

Pre-test

The validity of the aorta rings was confirmed by contraction with 0.3uM phenylephrine and then relaxation with 0.3 μM acetylcholine. If 70% or more relaxation was shown when comparing to the 0.3 μM Phenylephrine induced contraction, the aorta ring was defined to be suitable for further use as the endothelial layer remained intact for control group. And the aorta fail to give vasodilation response to 0.3 μM acetylcholine confirmed the absence of functional endothelium. Aorta ring was then equilibrated for an hour with three times changing Krebs solution before the test of samples.

Drug test

The aorta rings were contracted by 10nM U46619, which is a potent prostaglandin H_2 receptor agonist, for 20 minutes. Samples were then added to the organ bath and the vasomotion were monitored throughout the experiment. Samples were prepared by Krebs solution. For the nitric oxide synthase inhibitory group, 100 μM L-NNA was added and incubated for 30 minutes before adding U46619.

Measurement

Tensions were recorded by MacLab data acquisition system through Grass force-displacement transducers, FT-03E. Data was recorded by a sampling rate of 10 per second. The background noise of raw data was then removed by triangular (Bartlett) window type of smoothing with window size of 201 points width before analysis. Finally, results were expressed as percentage relaxation response of the maximal contraction induced by 10 nM U46619.

5.2.2 Results

Removal of endothelial layer

a) Danshen

No significant difference between the vasodilation of normal group with endothelium and the test group without endothelium under the treatment of 4 mg/ml Danshen was observed as shown in figure 5.8. The effect on the removal of functional endothelium could be observed in figure 5.8 that no relaxation was observed after addition of acetylcholine in the pre-test. After calculation, the results were expressed as percentage relaxation response of the maximal contraction induced by 10 nM U46619. The percentage relaxation against the time course was plotted as figure 5.9. Only 2 time points, namely 13^{th} and 14^{th} minute, showed significant differences while no significant differences between the two groups with and without endothelium at all other time points were observed. Therefore, the vasodilation effect of Danshen is not due to the presence of endothelium.

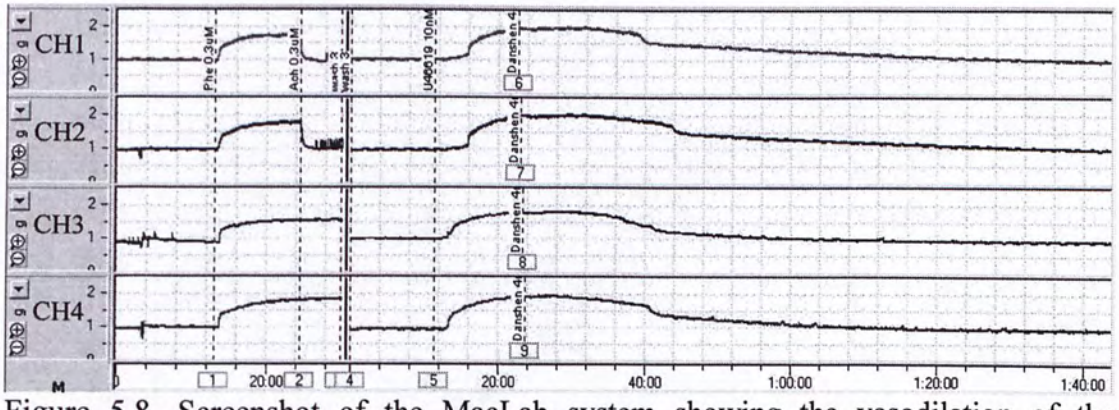


Figure 5.8. Screenshot of the MacLab system showing the vasodilation of the Danshen aqueous extract in the presence and the absence of endothelium. Vertical dimension represents the tension force of the aorta ring in the unit of gram (g) and the horizontal dimension represents the time course during the experiment. Channel 1 and 2 are normal aorta rings with intact endothelium while channel 3 and 4 are endothelium removed. The removal of functional endothelium could be observed in channel 3 and 4 that no relaxation was observed after addition of acetylcholine in the pre-test. Concentration of Danshen added to all channels was 4 mg/ml. No differences could be observed between 2 groups.

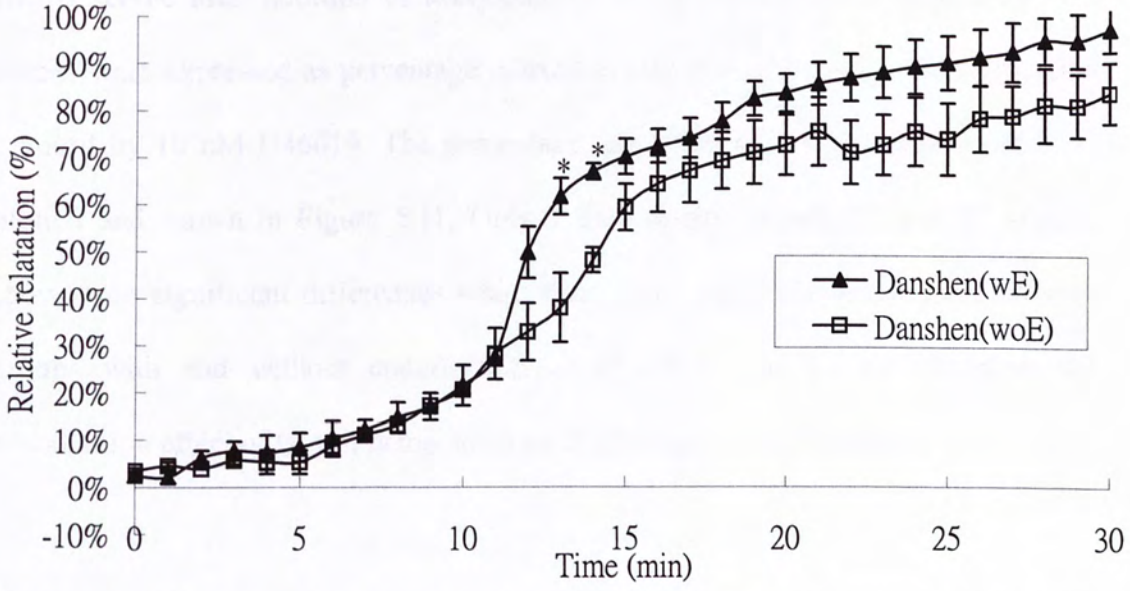


Figure 5.9. Endothelium independent vasodilation of Danshen aqueous extract. (wE) represents with endothelium, (woE) represents without endothelium. Data are mean ± SD of 4 aorta rings of each group. *P<0.05, compares with relaxation of the endothelium removed group.

b) Gegen

There are significant differences between the vasodilation of normal group with endothelium and the test group without endothelium under the treatment of 4 mg/ml Gegen extract was observed as shown in figure 5.10. The Group without endothelium did not show the vasodilation effect of Gegen extract after Gegen treatment. The effect of removal of endothelium could be observed in figure 5.10 that no relaxation was observed after addition of acetylcholine in the pre-test. After calculation, the results were expressed as percentage relaxation response of the maximal contraction induced by 10 nM U46619. The percentage relaxation against the time course was plotted and shown in Figure 5.11. Only 2 time points, namely 1st and 3rd minute, showed no significant differences while there were significant differences between groups with and without endothelium at all other time points. Therefore the vasodilation effect of Gegen is mediated by the presence of endothelium.

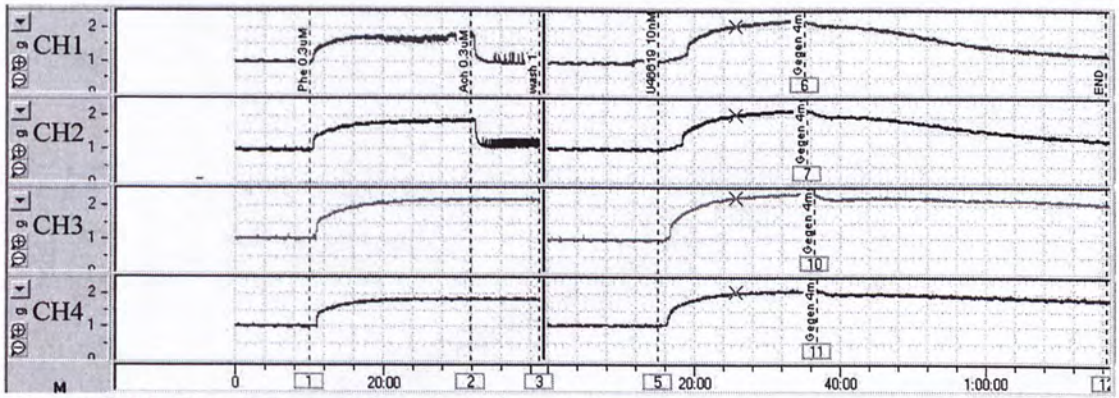


Figure 5.10. Screenshot of the MacLab system showing the vasodilation of the Gegen aqueous extract in the presence and the absence of endothelium. Vertical dimension represents the tension force of the aorta ring in the unit of gram (g) and the horizontal dimension represents the time course during the experiment. Channel 1 and 2 are normal aorta rings with intact endothelium while channel 3 and 4 are endothelium removed. The removal of functional endothelium could be observed in channel 3 and 4 that no relaxation was observed after addition of acetylcholine in the pre-test. Concentration of Gegen added to all channels was 4 mg/ml. Vasodilation effect was impaired in the endothelium removed group.

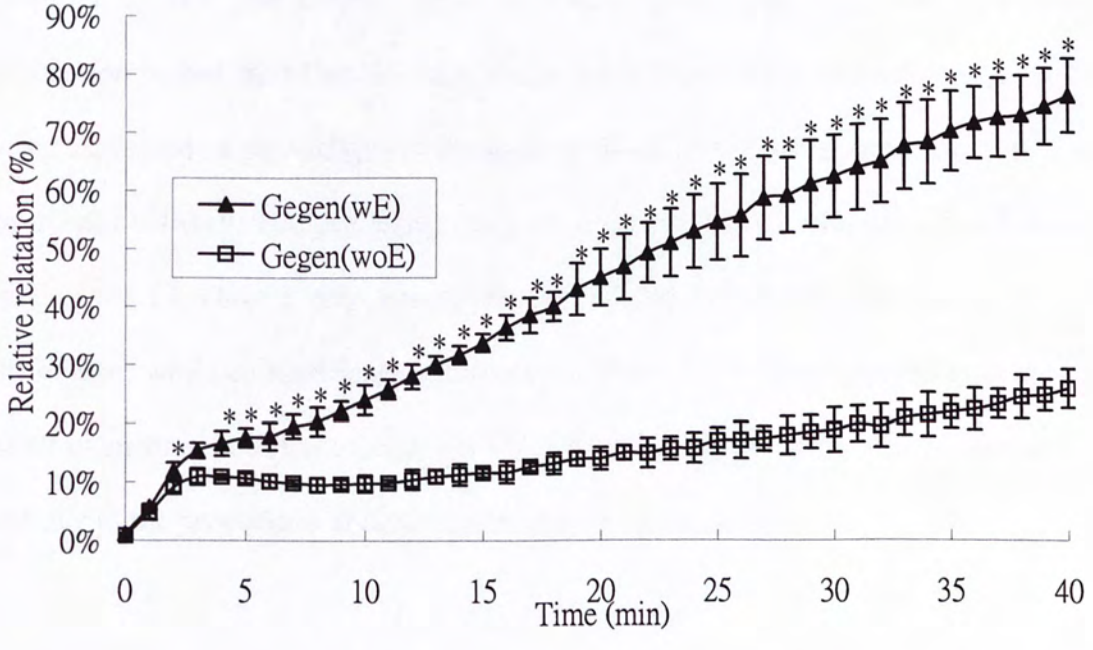


Figure 5.11. Endothelium dependent vasodilation of Gegen aqueous extract. (wE) represents with endothelium, (woE) represents without endothelium. Data are mean ± SD of 4 aorta rings of each group. *P<0.05, compares with relaxation of the endothelium removed group.

Nitric oxide synthase inhibitor, L-NNA

a) Danshen

No significant different in the vasodilation was observed between the normal group without nitric oxide synthase inhibitor and the test group with inhibitor, L-NNA, under the treatment of 4 mg/ml Danshen as shown in figure 5.12. The effect of addition of 100 μM L-NNA could be observed in figure 5.12 that 30 minutes incubation before the U46619 induced vasoconstriction. After calculation, the results were expressed as percentage relaxation response of the maximal contraction induced by 10 nM U46619. The percentage relaxation against the time was plotted and shown in figure 5.13. Only 2 time points, namely 7^{th} and 11^{th} minute, showed significant differences while no significant differences between with and without inhibitor groups at all other time points was observed. Therefore the vasodilation effect of Danshen is not due to the production of nitric oxide nitric by oxide synthase.

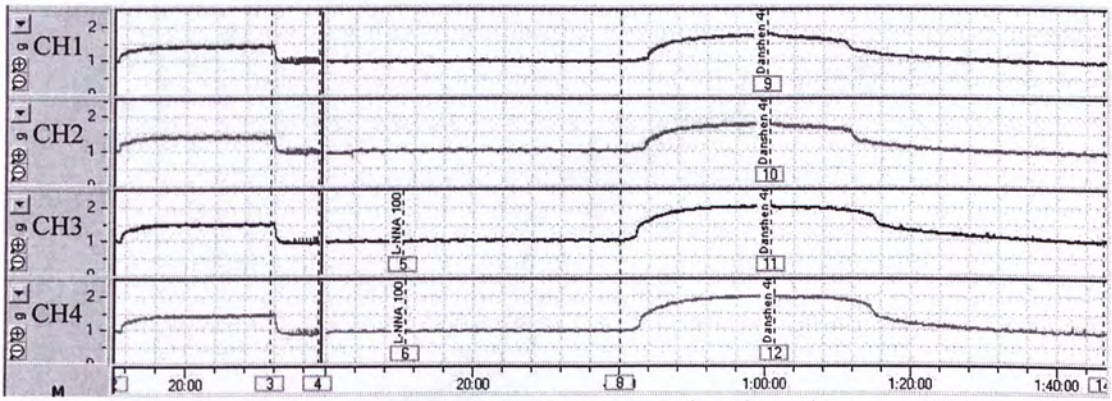


Figure 5.12. Screenshot of the MacLab system showing the vasodilation of the Danshen aqueous extract in the presence and absence of 100 μM L-NNA. Vertical dimension represents the tension force of the aorta ring in the unit of gram (g) and the horizontal dimension represents the time course during the experiment. Channel 1 and 2 are normal aorta rings without L-NNA while channel 3 and 4 are pre-treated with L-NNA for 30 minutes before test. Concentration of Danshen added to all channels was 4 mg/ml. No differences could be observed between 2 groups.

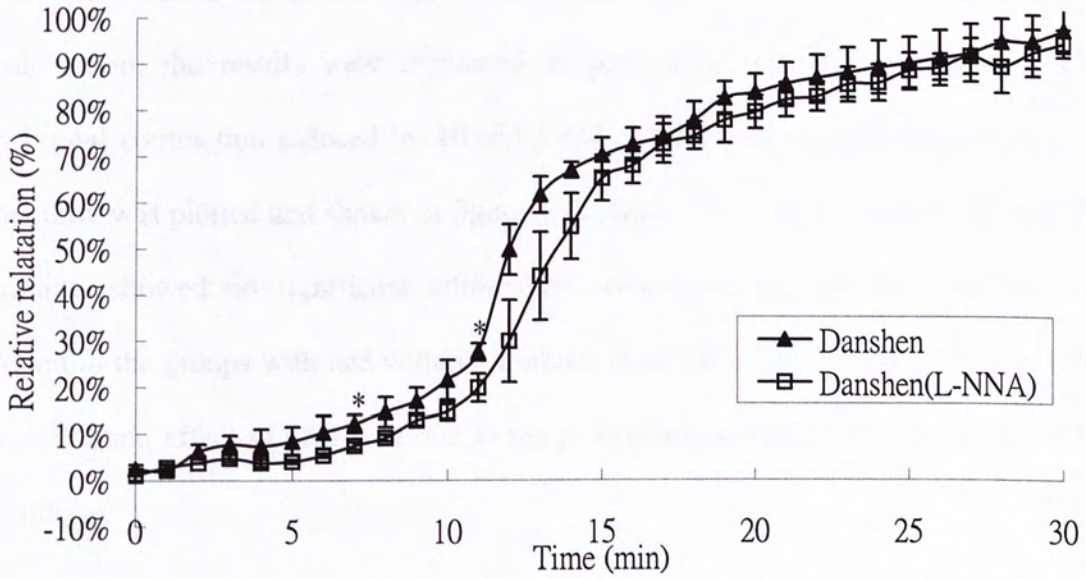


Figure 5.13. Nitric oxide synthase independent vasodilation of Danshen aqueous extract. Danshen (L-NNA) represents presence of 100 μM L-NNA, Danshen represents the absence of L-NNA. Data are mean ± SD of 4 aorta rings of each group. **P*-value<0.05, compares with relaxation of the endothelium removed group.

b) Gegen

There are significant differences between the vasodilation in the normal group without nitric oxide synthase inhibitor and the test group with inhibitor, L-NNA, with the treatment of 4 mg/ml Gegen extract as shown in figure 5.14. The Group with nitric synthase inhibitor, 100 μM L-NNA, impaired the vasodilation effect of Gegen extract. The effect of addition of 100 μM L-NNA could be observed in figure 5.14 that 30 minutes incubated before the U46619 induced vasoconstriction. After calculation, the results were expressed as percentage relaxation response of the maximal contraction induced by 10 nM U46619. The percentage relaxation against the time was plotted and shown in figure 5.15. Only 2 time points, namely 1st and 2nd minute, showed no significant differences while there are significant differences found in the groups with and without inhibitor at all other time points. Therefore, the vasodilation effect of Gegen is due to the production of nitric oxide by nitric oxide synthase.

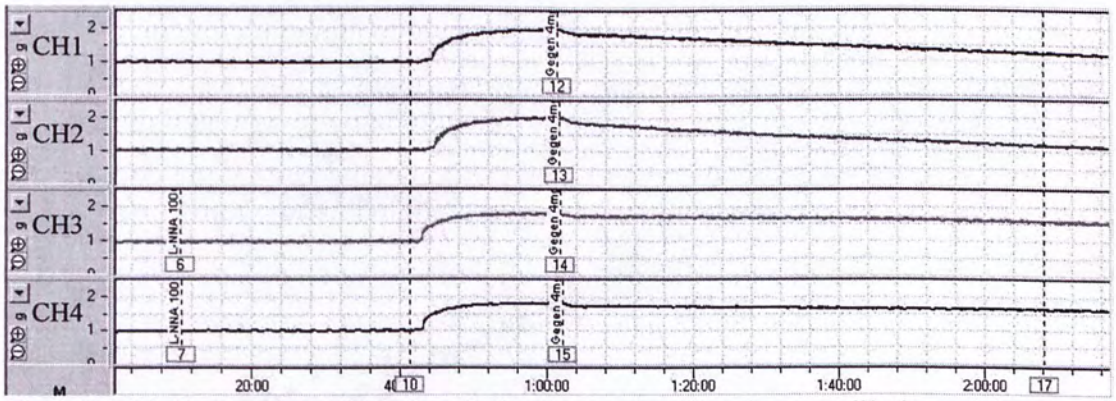


Figure 5.14. Screenshot of the MacLab system showing the vasodilation of the Gegen aqueous extract in the presence and absence of 100 μM L-NNA. Vertical dimension represents the tension force of the aorta ring in the unit of gram (g) and the horizontal dimension represents the time course during the experiment. Channel 1 and 2 are normal aorta rings without L-NNA while channel 3 and 4 are pre-treated with L-NNA 30 minutes before test. Concentration of Gegen added to all channels was 4 mg/ml. Vasodilation effect was impaired in the nitric oxide synthase group.

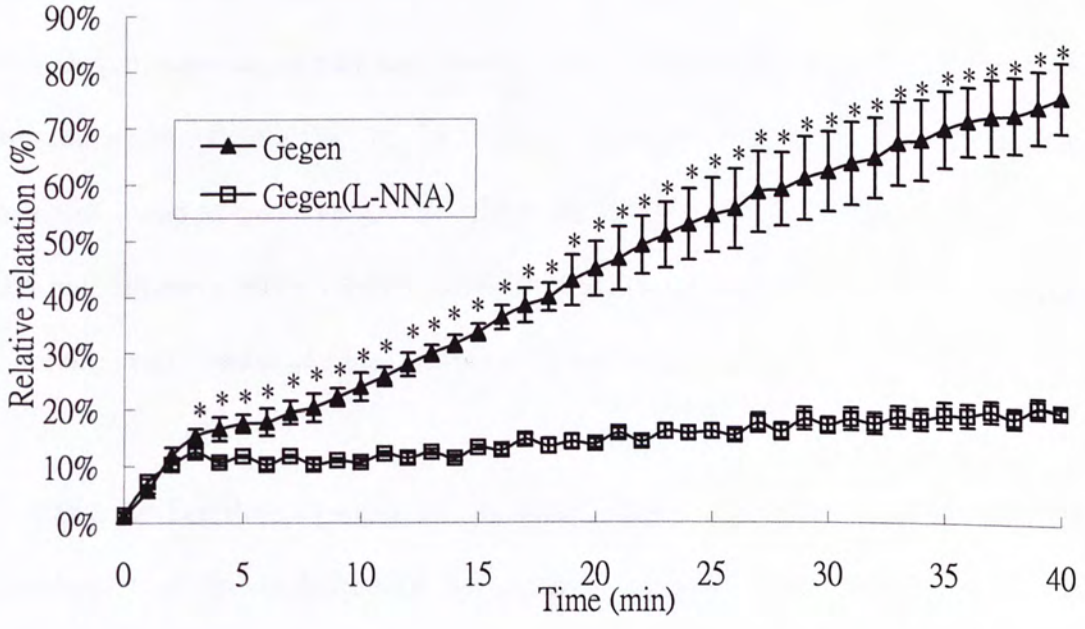


Figure 5.15. Nitric oxide synthase dependent vasodilation of Gegen aqueous extract. Gegen (L-NNA) represents presence of 100 μM L-NNA, Gegen represents the absence of L-NNA. Data are mean ± SD of 4 aorta rings of each group. *P<0.05, compares with relaxation of the endothelium removed group.

5.2.3 Discussion

Gegen aqueous extract exhibited endothelium dependent vasodilation as the removal of endothelial layer impaired the Gegen vasodilation effect. The finding that pre-treatment of nitric oxide synthase inhibitor also impaired the effect of Gegen aqueous confirmed that the vasodilation of Gegen are thought the up regulation of nitric oxide production by nitric oxide synthase. As many cardiovascular diseases are associated with an impairment of endothelium-dependent vasodilation, the finding that Gegen aqueous extract can stimulate the endothelium dependent vasodilation implied its beneficial effect to the cardiovascular diseases. In addition, nitric oxide was also found to have anti-platelet effect (Benjamin *et al.*, 1991 ; Moro *et al.*, 1996) and anti-adhesive effect (Kubes *et al.*, 1991), that is why Gegen was used to treat coronary heart disease and vascular diseases traditionally.

For the Danshen aqueous extract, result shows that the vasodilation effect of Danshen was an endothelium independent response. The possibility of the involvement of nitric oxide synthase can be excluded for Danshen.

Chapter 6 Anti-platelet study

People are very concerned platelet aggregation and thrombosis in cardiovascular diseases. They lead to deterioration of the blood vessels and complicated with atherosclerosis, myocardial infarction, stroke etc. Anti-platelet is a large topic in dealing with cardiovascular diseases.

Anti-platelet aggregation effects of Danshen and Gegen have been studied for a long time. A compound known as 2-isopropyl-8-methylphenanthrene-3,4-dione was isolated from Danshen which showed 30 times more potent than papaverine in anti-platelet aggregation when their IC50 were compared (Onitsuka *et al.*, 1983). Related diketophenanthrene derivatives such as tanshinone I, tanshinone II and cryptotanshinone were also found to have the inhibitory activity with less potency. Puerarin was isolated from Gegen which can elevate PGI_2 level. An increase in PGI_2 level results in an inhibition of platelet aggregation and improves blood flow (Yang *et al.*, 1990).

Apart from the anti-platelet aggregation effect, the effect of the 7:3 (D:G) compound formula on long-term platelet formation was unclear and was investigated in this study. The first model applied was colony-forming-unit megakaryocyte (CFU-MK) plasma clot colony assay.

6.1 CFU-MK plasma clot colony assay

Megakaryocyte (MK) is the progenitor cell of blood platelet. The bone marrow stem cells are capable to differentiate into different cell types under different cytokines stimulation during hematopoiesis. Thrombopoietin (TPO) is the key cytokine to stimulate the differentiation of MK cells and the formation of platelets. Therefore, CFU-MK plasma clot colony assay was applied to investigate the anti-platelet formation effect of 7:3 (D:G) compound formula, Danshen (D) and Gegen (G) aqueous extract.

6.2 Materials and Methods

Chemicals

Iscove's modified Dulbecco's medium (IMDM) was purchased from Life Technologies (Gibcobrl). Cytokines of recombinant Human IL-3 and thrombopoietin (TPO) were purchased from PeproTech. Fetal calf serum (FCS), 2-mercaptoethanol (2mE), bovine serum albumin (BSA), bovine plasma and calcium chloride ($CaCl_2$) were purchased from Sigma. Acetylcholine esterase (AChE) staining solution was made up of 0.5 mg/ml acetylthiocholine iodide, 100 mM sodium citrate, 3 mM cuppric sulfate, 0.5 mM potassium ferricyanate in 0.1 M PBS, pH 6.0.

Animal

Female six to eight weeks old BALB/C mice were killed by cervical dislocation. Femurs were removed and cut at both ends. Bone marrow was expelled with 2 ml IMDM by inserting 21- to 23-gauge needle into the end of femurs. Bone marrow cells

were dispersed and washed twice with IMDM by centrifugation at 300 x *g* for 10 minutes.

Plasma clot system

Bone marrow cells were cultured in 35 mm petri dishes using the plasma clot culture method. 2 x 10^5 cells were cultured in 1 ml system of IMDM with 10% FCS, 1% BSA, 10^{-4} M 2mE, 0.34 mg/ml $CaCl_2$, 10% bovine plasma, 20 ng/ml IL-3, 50 ng/ml TPO and various concentrations of samples. Dishes were incubated at 37 ℃ in a fully humidified atmosphere with 5% CO_2 for 7 days.

Acetylcholine esterase staining

The culture plates were fixed with 1 % paraformaldehyde in phosphate buffer (pH 6.0) for 15 minutes and dehydrated in the petri dishes. The fixed plates were then washed in phosphate buffer (pH 6.0). And then covered with acetylcholinesterase (AChE) staining solution for 4 hours at room temperature for identification of megakaryocyte colonies. Inverted microscopy was used for scoring the megakaryocyte colonies. A megakaryocyte colony was defined as a cluster of 3 or more AChE-positive cells as show in figure 6.1.

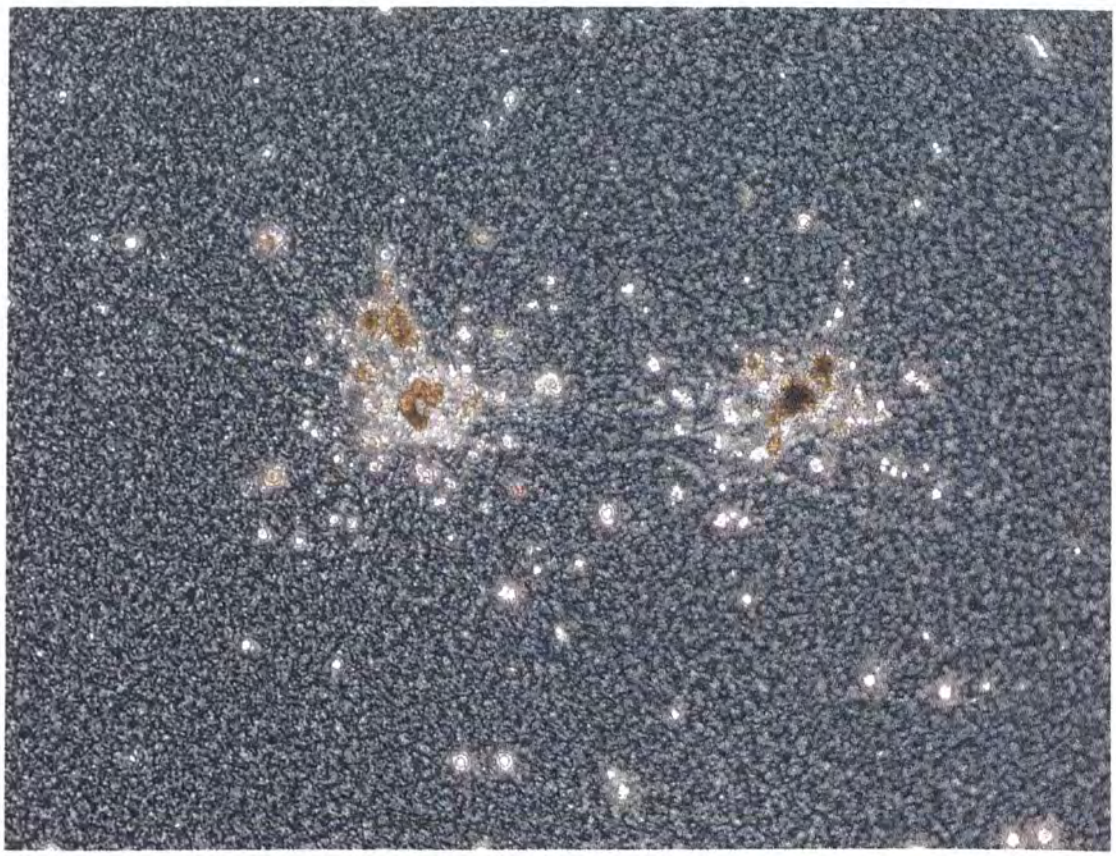


Figure 6.1. Two megakaryocyte colonies observed under inverted microscope. Where magnification is 400X.

6.3 Results

All of the three samples, namely 7:3 (D:G) compound formula, Danshen and Gegen aqueous extracts show inhibition to the MK cell proliferation. The MK colonies counts were shown in Figure 6.2 as a bar chart. The numbers of colonies of all three groups were found to drop gradually when the concentration increased from 50 μg/ml to 1000 μg/ml. IC50 was applied to compare their potency on the inhibition of MK cell proliferation (Figure 6.3). IC50 of Danshen, Gegen and 7:3 (D:G) formula were found to be 52 μg/ml, 97μg/ml and 161 μg/ml in a descending order respectively.

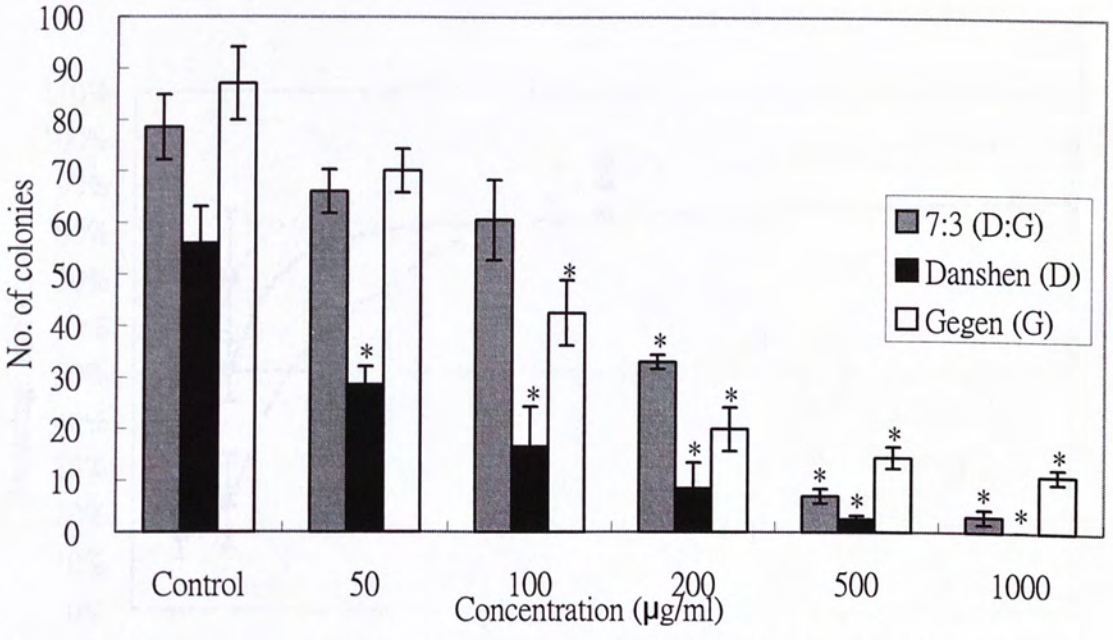


Figure 6.2. The number of MK colonies counts after 7 days incubation with 7:3 (D:G) compound formula, Danshen and Gegen in various concentrations. Data were presented by mean ± SD of 5 dishes each group. *P-value < 0.05 against control.

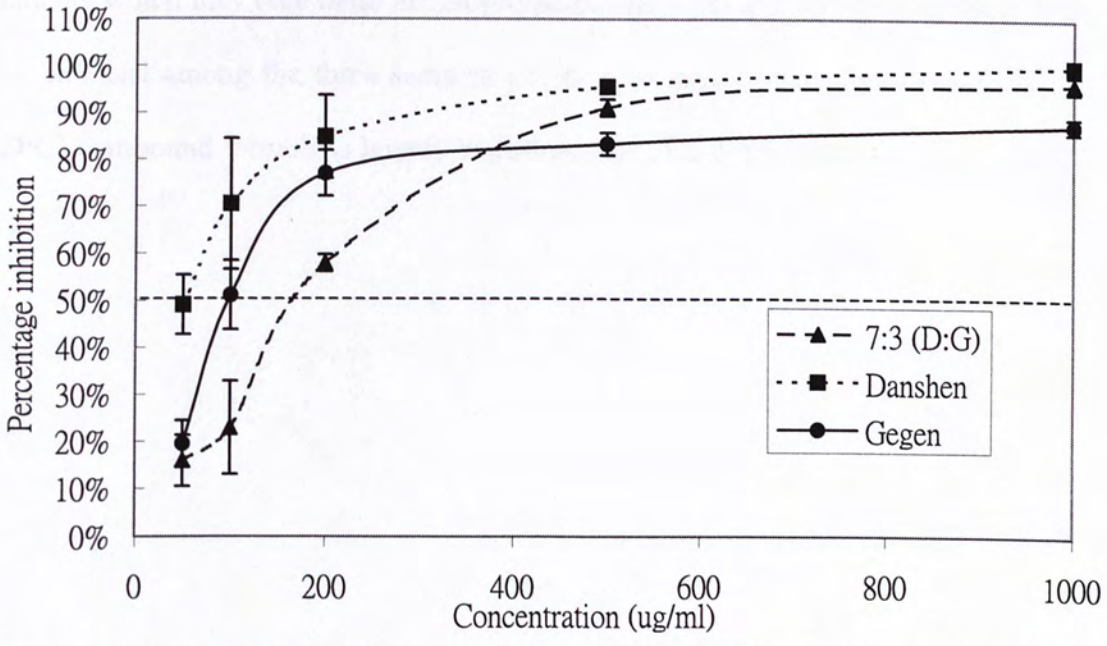


Figure 6.3. The IC50 of MK colonies inhibition by 7:3 (D:G) compound formula, Danshen and Gegen. IC50 of Danshen, Gegen and 7:3 (D:G) formula were found to be 52 μg/ml, 97 μg/ml and 161 μg/ml in descending order respectively. Data were presented by mean ± SD of 5 dishes each group.

6.4 Discussion

The inhibitory effect of 7:3 (D:G) formula, Danshen and Gegen on the megakaryocyte proliferation under the stimulation of TPO and IL-3 were demonstrated. As the MK cell proliferation was inhibited, the amount of platelets production would also be reduced. It is beneficial to the cardiovascular disease patients which they take these herbal products. The IC50 of Danshen was found to be the smallest among the three samples tested. This implies that the effect of the 7:3 (D:G) compound formula is largely contributed by effects of Danshen.

Chapter 7 Discussions and prospects

7.1 Discussions

The following findings were observed throughout my study:

a) The 7:3 (D:G) compound formula demonstrated antioxidative effect against AAPH induced RBC hemolysis with IC50 of 168 μg/ml.

b) The 7:3 (D:G) compound formula protected *ex-vivo* ischemia reperfusion heart to have 60% contractile force recovery and reduced the heart damage which was reflected by tissue specific enzyme LDH and CK assays.

c) The 7:3 (D:G) compound formula caused vasodilation to the pre-contracted *ex-vivo* aorta and synergistic effect of compound formula was shown.

d) Danshen extract showed endothelium independent vasodilation, while the vasodilation effect of Gegen extract was found to be endothelium dependent and the mechanism is via the function of nitric oxide synthase.

e) The 7:3 (D:G) compound formula inhibited MK cell proliferation at IC50 of 161 μg/ml.

The above-mentioned findings in our study imply that 7:3 (D:G) compound formula is beneficial to cardiovascular disease patients as it showed antioxidative effects, vasodilation effect and anti-platelet formation effect.

The cardiovascular tonic effect of 7:3 (D:G) compound formula was demonstrated in three areas of antioxidant, vasodilation and anti-platelet formation. However, the antioxidative effect is mainly due to Danshen. Antioxidant quenches the oxidative reactive species in the blood stream which lower the chance of oxidizing LDL formation. It would reduce the chance of atherosclerosis formation and its other complicated adverse effects. Danshen and Gegen exert vasodilation via endothelium independent and dependent mechanism respectively. Endothelium dependent vasodilation increased endogenous nitric oxide to the smooth muscle cells. Lower blood pressure would not only reduce the chance of development of stroke and myocardial infarction, but also reduce morbidity and mortality of cardiovascular diseases. Danshen and Gegen are demonstrated to have different effects on different areas studied by us in this thesis. It supports that herbal practitioners view people as ecosystem in miniature and use compound formula to improve one's capacity to balance and renew his resources. The different TCM components in the compound formula affect different organs to give the final outcome of body resources balanced.

For the compound formula research, 7:3 (D:G) compound formula shows enhanced extraction efficiency of Salvianolic acid B when comparing to individual Danshen (as mentioned in chapter 3). Therefore each component TCM of a compound formula may play a very important role in affecting the extraction efficiency on each other component TCM. It further supports the validity of our methodology that the compound formulae were prepared by mixed TCMs decoction but not mixing extract of individual TCM. It is a more precise method to study the compound formulae of Chinese Medicines as TCMs are always decocted by a mixture of herbs. Further

experiments should be done on comparing the chemical species of the compound formulae with individual TCMs. In order to investigate the additive effect of TCMs, the TCMs should be used in compound formula to enhance or neutralize another component TCM.

In the figure 5.7 in chapter 5, it showed the synergistic effect of combined use of Danshen and Gegen. The benefit of the 7:3 (D:G) compound formula has been definitely confirmed although the underlying mechanism is still unknown. These findings and methodologies are not only valuable for the further study of the Danshen-Gegen compound formula, but also useful for other TCM compound formula studies in which combined formula with different herbal materials may be included.

7.2 Prospects

7.2.1 The 7:3 (D:G) compound formula capsule with GMP

The 7:3 (D:G) compound formula capsules as shown in figure 7.1, 500 mg extract encapsulated each, were manufactured with compliance to GMP by Hong Kong Institution of Biotechnology (HKIB). It is a very good milestone to make TCM to be accepted in Western countries. GMP is a part of quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorization. Raw material should be harvested at the best birthplace and period, and should be authenticated to contain no adulterants and substitutes. They are ensured to be free of heavy metals, remaining pesticides and micro-organisms. The materials should be examined by visual and chemical parameters. The amount of active ingredients should be monitored by bioassays.

As GMP was the international recognitions and guaranteed the quality, it is pivotal to the international market. In addition, TCM in capsule form brings benefits to users to reduce the amount of intake and save time for decoction.

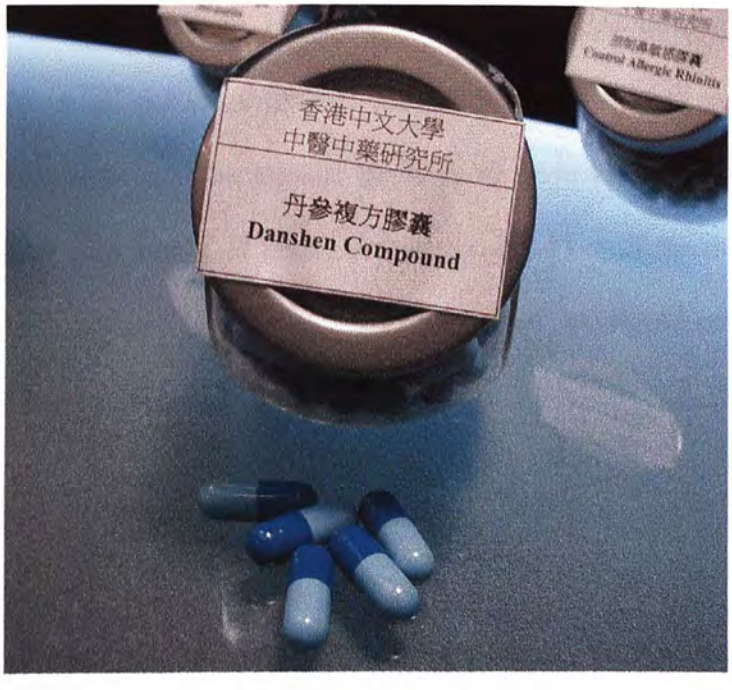


Figure 7.1. The 7:3 (D:G) compound formula capsules manufactured by HKIB.

7.2.2 Clinical trial of the capsule

The approval of Clinical Research Ethical Committee on the issue of performing clinical trial of Danshen to Gegen in 7:3 ratio compound formula was obtained. Double blind test with placebo method was employed. The clinical trial was started in May 2002 at Prince of Wales Hospital, Hong Kong. Secondary prevention was studied first where all participants have cardiovascular disease records that at least one vessel shows 50% atherosclerosis. Participants have to intake 6 capsules daily (manufactured in compliance of GMP, see section 7.2.1), 3 in the morning and 3 in the night, for a 24-weeks period and their cases were monitored once a week. The clinical trial is still in progress.

References

Benjamin N., Dutton J.A.E. and RitterJ.M. (1991). Human vascular smooth muscle cells inhibit platelet aggregation when incubated with glyceryl trinitrate: evidence for generation of nitric oxide. Br J Pharmacol. 102, 847 - 850.

Buda C., Sallanon M. and Jin G.Z. (1984). Effect of l-tetrahydropalmatine on sleep-waking cycle of cats. Zhongguo Yao Li Xue Bao. 5, 5-8.

Chai X.S., Wang Z.X., Chen P.P., Wang L.Y., Lu X.R. and Kang B. (1985). [Anti-arrhythmic action of puerarin]. Zhongguo Yao Li Xue Bao. 6, 166-8.

Cochrane C.G. (1991). Mechanisms of oxidant injury of cells. Mol Aspects Med. 12, 137-47.

Duffy S.J., O'Brien R.C., New G., Harper R.W. and Meredith I.T. (2001). Effect of anti-oxidant treatment and cholesterol lowering on resting arterial tone, metabolic vasodilation and endothelial function in the human forearm: a randomized, placebo-controlled study. Clin Exp Pharmacol Physiol. 28, 409-18.

Fan L.L., Zeng G.Y., Zhou Y.P., Zhang L.Y. and Cheng Y.S. (1982). Pharmacologic studies on Radix puerariae: effects of puerariae flavones on coronary circulation, cardiac hemodynamics and myocardial metabolism in dogs. Chin Med J (Engl). 95, 145-50.

Fan S., Sun L., Wu Y., Zhao H. and Zhang F. (1997). [Effects of both puerarin and gypsum on the firing of pyrogen-treated thermosensitive neurons in the region POAH of anesthetized cats]. Zhongguo Ying Yong Sheng Li Xue Za Zhi. 13, 71-4.

Feng Y.P., Zhang L.Y. and Zeng G.Y. (1984). [Effect of puerarin and analogues of daidzein on adrenoceptors]. Zhongguo Yao Li Xue Bao. 5, 238-41.

Furchgott R.F. and Zawadzki J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 288, 373 - 376.

Halliwell B. (1997). Antioxidants and human disease: a general introduction. Nutr Rev. 55, 44-52.

Hearse DJ. (1994). Myocardial ischaemia: can we agree on a definition for the 21st century? Cardiovasc Res. 28, 1737-44.

Ho C.T. and Zheng Q.Y. (2002). Quality management of nutraceuticals. Washington, DC: American Chemical Society.

Hu J., Xie J., Hu J., Zhang Y., Wang J. and Chen R. (1994). [Effect of some drugs on electroacupuncture analgesia and cytosolic free Ca2+ concentration of mice brain]. Zhen Ci Yan Jiu. 19, 55-8.

Huang P.L., Huang Z., Mashimo H., Bloch K.D., Moskowitz M.A., Bevan J.A. and Fishman M.C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase. Nature. 377, 239 - 242.

Huang T.K. and Shi C. (1996). Zhong yao fang ji xian dai yan jiu da dian. Beijing: Ke xue chu ban she.

Ji X., Tan B.K., Zhu Y.C., Linz W. and Zhu Y.Z. (2003). Comparison of cardioprotective effects using ramipril and DanShen for the treatment of acute myocardial infarction in rats. Life Sci. 73, 1413-26.

Jin G.Z., Xu J., Zhang F.T., Yu L.P., Li J.H. and Wang X.L. (1983). [Relevance of the sedative-tranquilizing effect of l-tetrahydropalmatine to brain monoaminergic neurotransmitters]. Zhongguo Yao Li Xue Bao. 4, 4-10.

Kang D.G., Yun Y.G., Ryoo J.H. and Lee H.S. (2002). Anti-hypertensive effect of water extract of danshen on renovascular hypertension through inhibition of the renin angiotensin system. Am J Chin Med. 30, 87-93.

Kannel W.B. (2000). Fifty years of Framingham Study contributions to understanding hypertension. J Hum Hypertens. 14, 83 - 90.

Keung W.M. and Vallee B.L. (1998). Kudzu root: an ancient Chinese source of modern antidipsotropic agents. Phytochemistry. 47, 499-506.

Kubes P., Suzuki M. and Granger D.N. (1991). Nitric oxide: An endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci USA. 88, 4651 - 4655.

Luscher T.F. and Noll G. (1995). The pathogenesis of cardiovascular disease: role of the endothelium as a target and mediator. Atherosclerosis. 118, 81-90.

Manning A., Bernier M., Crome R., Little S. and Hearse D. (1988). Reperfusion-induced arrhythmias: a study of the role of xanthine oxidase-derived free radicals in the rat heart. J Mol Cell Cardiol. 20, 35-45.

McCord J.M., Roy R.S. and Schaffer S.W. (1985). Free radicals and myocardial ischemia. The role of xanthine oxidase. Adv Myocardiol. 5, 183-9.

Moncada S. and Higgs A. (1993). Mechanisms of disease: the L-arginine - nitric oxide pathway. New Engl J Med. 329, 2002 - 2012.

Moncada S., Palmer R.M. and Higgs E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev. 43, 109-42.

Moro M.A., Russel R.J., Cellek S., Lizasoain I., Su Y., Darley-Usmar V.M., Radomski M.W. and Moncada S. (1996). cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. Proc Natl Acad Sci USA. 93, 1480–1485.

Onitsuka M., Fujiu M., Shinma N., Maruyama H.B. (1983). New platelet aggregation inhibitors from Tan-Shen; radix of Salvia miltiorrhiza Bunge. Chem. Pharm. Bull. 31, 1670-5.

Palmer R.M., Ferrige A.G. and Moncada S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature. 327, 524-6.

Radomski M.W. and Moncada S.(1993). Regulation of vascular homeostasis by nitric oxide. Thromb Haemost. 70, 36–41.

Rossi G.P., Seccia T.M. and Nussdorfer G.G. (2001). Reciprocal regulation of endothelin-1 and nitric oxide: relevance in the physiology and pathology of the cardiovascular system. Int Rev Cytol. 209, 241 - 272.

Rubanyi G.M., Romero J.C. and Vanhoutte P.M. (1986). Flow-induced release of endothelium-derived relaxing factor. Am J Physiol. 250, 1145-9.

Ryu S.Y., Lee C.O. and Choi S.U. (1997). In vitro cytotoxicity of tanshinones from Salvia miltiorrhiza. Planta Med. 63, 339-42.

Soji Y., Kadokawa T., Masuda Y., Kawashima K. and Nakamura K. (1969). [Effects of Corydalis alkaloid upon inhibition of gastric juice secretion and prevention of gastric ulcer in experimental animals]. Nippon Yakurigaku Zasshi. 65, 196-209.

Song X.P., Chen P.P. and Chai X.S. (1988). [Effects of puerarin on blood pressure and plasma renin activity in spontaneously hypertensive rats]. Zhongguo Yao Li Xue Bao. 9, 55-8.

Spence J.D. (1984). Hemodynamic effects of antihypertensive drugs. Possible implications for the prevention of atherosclerosis. Hypertension. 6, 163-8.

Spence J.D., Perkins D.G., Kline R.L., Adams M.A. and Haust M.D. (1984). Hemodynamic modification of aortic atherosclerosis. Effects of propranolol vs hydralazine in hypertensive hyperlipidemic rabbits. Atherosclerosis. 50, 325-33.

Stamler J.S., Loh E., Roddy M.A., Currie K.E.and Creager M.A. (1994). Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. Circulation. 89, 2035 - 2040.

Suematsu M., Schmid-Schonbein G.W., Chavez-Chavez R.H., Yee T.T., Tamatani T., Miyasaka M., Delano F.A. and Zweifach B.W. (1993). In vivo visualization of oxidative changes in microvessels during neutrophil activation. Am J Physiol. 264, 881-91.

Tseng K.Y., Chou Y.P., Chang L.Y. and Fang L.L. (1974). [Pharmacologic studies of Radix puerariae. I. Its effect on dog arterial pressure, vascular reactivity, and cerebral and peripheral circulation]. Zhonghua Yi Xue Za Zhi. 5, 265-70.

von zur Muhlen B., Kahan T., Hagg A., Millgard J. and Lind L. (2001). Treatment with irbesartan or atenolol improves endothelial function in essential hypertension. J Hypertens. 19, 1813-8.

von zur Muhlen B., Millgard J. and Lind L.(2001). Divergent effects of different beta-blocking agents on endothelium-dependent vasodilatation in the human forearm. Blood Press. 9, 287-92.

Wang B.S., Wang L.J., Zhang Y.B., Lu J.S., Tang N., Huang Y.T., Yan W.H. and Song W. (1997). Reduction of myocardial ischemia-reperfusion injury by isovolumic hemodilution. Clin Hemorheol Microcirc. 17, 181-6.

Ward PA. (1991). Mechanisms of endothelial cell injury. J Lab Clin Med. 118, 421-6.

Wu Y.J., Hong C.Y., Lin S.J., Wu P. and Shiao M.S. (1998). Increase of vitamin E content in LDL and reduction of atherosclerosis in cholesterol-fed rabbits by a water-soluble antioxidant-rich fraction of Salvia miltiorrhiza. Arterioscler Thromb Vasc Biol. 18, 481-6.

Xue Q.F. (1986). [Effect of ligustrazine and salvia miltiorrhiza on microcirculation in the hamster cheek pouch]. Zhonghua Yi Xue Za Zhi. 66, 334-7.

Yang G., Zhang L. and Fan L. (1990). [Anti-angina effect of puerarin and its effect on plasma thromboxane A2 and prostacyclin]. Zhong Xi Yi Jie He Za Zhi. 10, 82-4.

Yue K.L. (1981) [Analgesic and antispastic effects of protopine]. Zhongguo Yao Li Xue Bao. 2, 16-8.

Zhong R.X., Shi R.R., Huang L.X. Liu H. Tao J.Y. and Zhu X.Y. (1986). Spasmolytic effect of protopine and Corydalis decumbens on isolated cat ciliary muscle and guinea pig ileum. Chinese Traditional and Herbal Drugs. 17, 303-306.

孫永智. (1986). 丹參葛根元胡片治療冠心病 40 例臨床療效觀察. 北京中醫雜誌. 5, 25-26.